A STUDY OF THE ASCORBIC ACID INTAKE REQUIRED TO MAINTAIN TISSUE SATURATION IN NORMAL ADULTS

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ONE FIGURE

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A number of attempts have been made to estimate the vitamin C requirement of human beings. This subject has been recently reviewed by Smith ('38). According to this author, three levels of requirement may be distinguished: the physiologically indispensable, the adequate, and the saturation or luxus consumption values. The comment has recently been made (Current Comment, '38) that "It may well be that the varying results of different laboratories may be brought into harmony if distinction is made between actual requirement and indispensable minimum, the latter being the amount that is sufficient to prevent scurvy and the former also including that quantity of vitamin C necessary for normal functioning of all body processes which may require or be influenced by vitamin C." In the case of substances which are readily excreted when taken in excess of bodily needs the assumption is often made that a desirable allowance is that amount which produces maximum retention. Although there is at present no clear evidence that it is desirable to maintain the tissues is a constant state of saturation with respect to vitamin C, it is obvious that the daily requirement under ordinary circumstances cannot be greater than the minimum amount which will maintain such a state of saturation.

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These experiments were designed to determine the smallest ascorbic acid intake which would maintain the tissues in a fully saturated state with respect to vitamin C and thus to set an upper limit to the amount of ascorbic acid which the adult can use under normal conditions of health and activity.

EXPERIMENTAL

Subjects

Seven normal adults, five women and two men, served as subjects for this study. Table 1 gives information concerning the heights, weights and ages of the subjects at the beginning of the experiment and their basal metabolic rates

TABLE 1

The age, sex, height, weight and basal metabolism of each subject

SUBJECT	AGE	SEX	HEIGHT IN INCHES	INITIAL WEIGHT IN POUNDS	BASAL METABOLISM IN CALORIES PER 24 HOURS
A	38	Female	61	140	1340
B	37	Female	62	130	1390
C	23	Female	64½	115	1435
D	22	Female	68¼	135	1160
E	34	Female	70½	167	1700
F	24	Male	72½	160	1670
G	23	Male	72¾	159	1820

expressed in calories. Subjects F and G gained several pounds in the course of the experiment, subject B usually lost 2 or 3 pounds at the beginning of an experimental period, and the others tended to maintain their original weights or to fluctuate only slightly.

Because of the possible effect of activity on vitamin C metabolism, severe physical exercise was avoided, and the subjects recorded any unusual activity.

Methods

Diet. The basal diet used in the study was a modification of that used in a previous experiment (O'Hara and Hauck,

'36). The following foods were used ad libitum: a whole wheat cereal with added wheat germ, butter, brown and refined sugar, Ry Krisp, whole wheat and white flour, pecans, cooking fat, rice and coffee. Each subject had an egg each day, and eggs were used to a limited extent in cooking, for example in making cookies. The following foods were used in weighed amounts either because they contained traces of vitamin C or because a certain intake was considered advisable to insure the adequacy of the diet: 100 gm. of canned beets plus 10 gm. of juice, 100 gm. of canned diced carrots plus 10 gm. of juice, 100 gm. of cooked dried prunes plus 25 gm. of juice, 100 gm. of canned pears plus 50 gm. of juice, 60 gm. of evaporated milk, 100 gm. of ground beef and 60 gm. of Cheddar cheese. The average amount of vitamin C furnished by the basal diet was approximately 10 mg. daily, as estimated from indophenol titration. An ascorbic acid supplement [1] dissolved in a small amount of water was given daily at breakfast. The 400 mg. test dose was divided equally between the morning and noon meals.

Urine collections. Since in fifty-eight out of sixty-four periods, urine titrations were made twice daily, in the morning and early evening, day and night specimens were collected separately. The day sample included the urine collected at the first voiding after breakfast and throughout the day. Night collections included urine voided from the evening through the first voiding after rising. Each subject collected this last specimen at approximately the same time each morning.

Each urine specimen was divided into two equal parts, one of which was placed in a brown bottle and was acidified immediately with 10% by volume of 2 N sulfuric acid containing 2% of metaphosphoric acid. This portion was used for the estimation of vitamin C excretion, whereas the remaining portion was kept for the pH determination. Both portions

[1] Synthetic ascorbic acid (Hoffman La Roche) was used except during the period when subjects C, D and E received a 25 mg. supplement, at which time Merck's Cebione tablets were used.

were stored in the refrigerator. Preliminary observations indicated that specimens of urine preserved in this manner lost little vitamin C in 24 hours. In nine observations, the loss ranged from 0 to 6%, with a mean loss of less than 2%.

Determination of vitamin C in urine. The vitamin C content of the urine samples was determined by titration with a buffered solution of the indicator, sodium 2,6-dichlorobenzenone-indophenol,[2] standardized every other day against a known solution of ascorbic acid.

The indicator was run quickly from a microburette into a measured portion of the acidified, refrigerated urine. Either 2 ml. or 5 ml. aliquot portions were used depending upon the concentration of the urine and the amount of vitamin C it was expected to contain.

All acid solutions used for preserving the urine, the solutions of indicator and of ascorbic acid for standardizing the dye were made up with water redistilled from glass.

Determination of urinary pH. The pH of 24-hour specimens of urine was determined colorimetrically, using the La Motte Universal pH outfit.

Plan of the experiment

Before beginning any experimental period, the subjects were saturated by giving them large doses of vitamin C, usually in the form of the juice of 4 to 8 oranges daily for 3 days in addition to the usual diet. The urine was collected and titrated on the day preceding the experimental period, to be sure that the subject was saturated. With the exception of a few instances preceding saturation periods when supplements of 200 mg. of ascorbic acid were given, the urinary excretion on this day did not fall below 190 mg. of ascorbic acid. In the majority of instances the excretion of ascorbic acid on the day preceding the experimental period was between 200 and 300 mg.

The response to a 400 mg. test dose following a 4- or 5-day period during which a 200 mg. supplement of vitamin C was

[2] Eastman Kodak Company.

given daily, was taken as the criterion for complete saturation. At least three such periods were studied for all subjects.

Similar test doses were given following 6-day periods on various lower levels of ascorbic acid intake and the responses were compared with those obtained when the subject was known to be saturated. If the response to the test dose equaled or exceeded the lowest response by the same subject following a period when the supplement was 200 mg. daily, the subject was considered to be saturated. Thus each subject provided his own individual standard for comparison.

In earlier experiments it was noted that if a subject had been previously saturated, the level of excretion of ascorbic acid was approximately constant after the second or third day on a constant intake. It was assumed therefore that a 6-day period would allow sufficient time for adjustment to a given level of intake, and with few exceptions, this was the period used.

The results presented here represent a total of sixty-four experimental periods, or from seven to twelve periods for each subject, covering a total time of from 5 to 17 months.

RESULTS AND DISCUSSION

Requirement to maintain tissue saturation

The values reported as ascorbic acid excretion represent total indophenol-reducing substances present. The enzymic method suggested by Tauber and Kleiner ('35) was used to determine what effect the basal diet might have on the urinary output of reducing substances other than vitamin C. In twenty-one determinations, for six subjects, there were only three instances in which the non-specific reducing capacity of the urine exceeded 3 mg. per day. The average value was 1.6 mg. As judged by this method, therefore, the amount of reducing substance other than vitamin C was too small to affect the interpretation of results. In any case, the amount of non-specific reducing substance in the urine could not have exceeded the total excretion of indophenol-reducing substance

in a previous experiment (O'Hara and Hauck, '36) in which the subjects excreted 9 to 12 mg. daily during the fourth week on the basal diet only.

The urinary responses to the 400 mg. test dose following saturation periods during which a daily supplement of 200 mg. of ascorbic acid was taken in addition to the basal diet are shown in table 2 (last column). It will be noted that subjects A and G gave no individual response above 249 mg., and that subjects B and C gave none lower than 240 mg. Individual differences in behavior have been observed by other authors, notably Wachholder and Hamel ('37) who relate them to differences in kidney threshold.

Subjects A, D, E and F showed a fairly regular steplike increase in response to the test dose with increasing levels of intake, until a value characteristic of saturation was reached (fig. 1 and table 2). Subject C's responses to the test dose were somewhat more irregular, her excretion following the 60 mg. level being 158 mg. as compared to 179 mg. following the 35 mg. level of intake. The low response of this subject following the 110 mg. level was probably due to diarrhea. No explanation is apparent for the variable behavior of subject G in response to the test doses.

The responses of subject B to test doses following levels of intake from 35 to 85 mg. were remarkably similar. Since marked differences between the 35 and 60 mg. levels were apparent when the experimental period was continued for 2 weeks instead of 6 days, it appears that the 6-day experimental period was not long enough for this subject to show small differences in tissue saturation. Note that this subject tended to give a high response to the test dose following saturation periods. Such behavior would be expected if the kidney threshold was low, but too few data are available on the plasma level of vitamin C for this subject to indicate whether this is the explanation.

The data in table 2 indicate that a minimum intake of between 70 to 85 mg. of ascorbic acid was necessary to maintain tissue saturation in subjects C and D under the conditions

TABLE 2

Twenty-four hour excretion of ascorbic acid in response to the test doses, following periods on various levels of ascorbic acid intake

SUBJECT	DAILY INTAKE OF ASCORBIC ACID IN MILLIGRAMS												
	10	35	60	70	80	85	90	95	100	110	125	135	210
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
A				133	191	204	201		266				222, 227, 248
B	141	191, 107[1]	189[1]	204	203	201			254				242, 274, 306
C		179	158[2]	239		253				196[3]			240, 269, 281
D		117	215[2]	215		247				251			228, 256, 275
E		62	140[2]			177		201, 207	232, 204[1]				200, 210, 228, 278
F									160[4]	178	210	245[4]	222, 210, 232
G			107			172			139	216, 237[4]	167	225[4]	209, 241, 249

[1] Subject had been on this level of intake for 14 days instead of 6 days before the test dose.
[2] Subject had been on this level 4 days only, before the test dose.
[3] Subject suffered from diarrhea on this day.
[4] Determination made on pooled 24-hour specimens.

of this experiment. Subject A required between 90 and 100 mg., subject B between 85 and 100 mg. and subject E about 95 to 100 mg., just to maintain tissue saturation. The male subjects, F and G, apparently required more vitamin C, although they were of approximately the same size as subject E. Subject F required between 125 and 135 mg. of vitamin C daily to maintain saturation, and although subject G's responses were more variable, his requirement appeared to be between 110 and 135 mg. When calculated on the basis of the lower figure in each case, the requirement per kilogram of body weight ranged from approximately 1 mg. for subject D to 1.6 mg. for subject F. This would represent approximately 60 to 96 mg. for an adult weighing 60 kg. If the upper figure is used as a basis for calculation, the variation is from approximately 1.2 to 1.7 mg. per kilogram or from 72 to 102 mg. for an adult weighing 60 kg. Thus, as a recent review has pointed out (Smith, '38) the least amount of vitamin C which will maintain tissue saturation is several times the amount estimated as the physiologically indispensable minimum.

According to Smith ('38) ". . . . a comparison of the absolute and relative values for different ages suggests that vitamin C requirements are related both to body weight and to metabolic rate, the effect of the latter being the more pronounced." For this reason, basal metabolic rates of the subjects are recorded (table 1). In general there is a rough correspondence between the basal metabolism in calories and the amount of vitamin C required to maintain saturation. It is possible that there may be a more direct relation between total energy metabolism and vitamin C requirement.

It is of interest that the amount of vitamin C necessary just to maintain tissue saturation, as determined in these experi-

Fig. 1 Urinary excretion of ascorbic acid for seven subjects on various levels of ascorbic acid intake. In most instances where data were available for more than one period on the same level of intake, one period only is represented. The saturation period shown for each subject is the one in which the response to the test dose was lowest. x indicates that the urine specimen was not available for analysis; y, a day on which the subject had diarrhea; z, a period during which no ascorbic acid supplement was given.

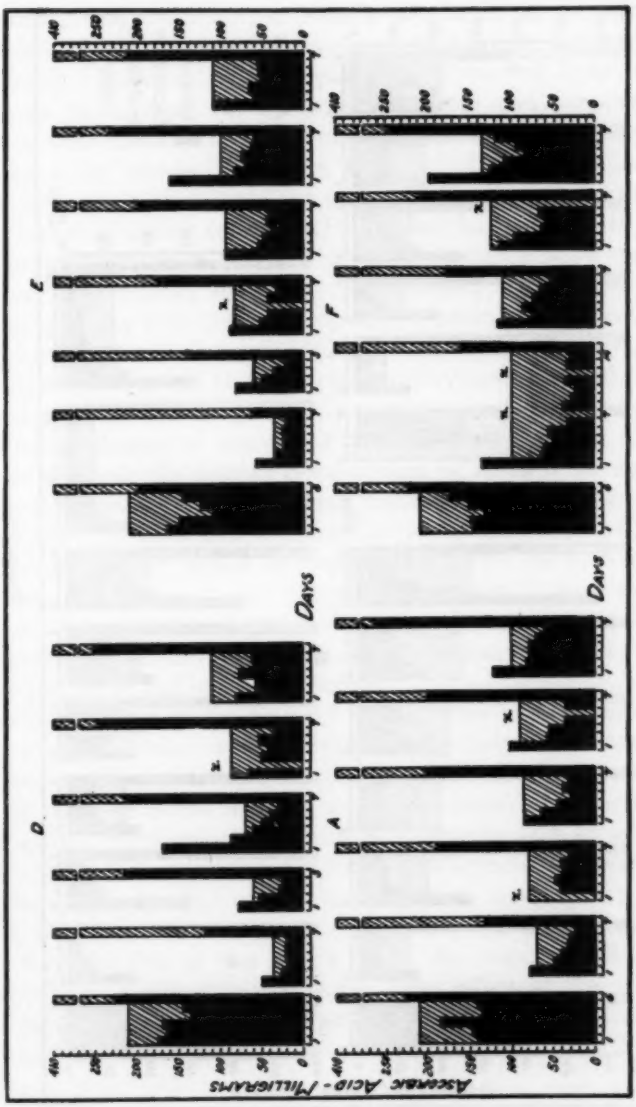
ASCORBIC ACID - MILLIGRAMS
400
250
200
150
100
50
0
A
D
E
F
Days
Days

Figure 1 a

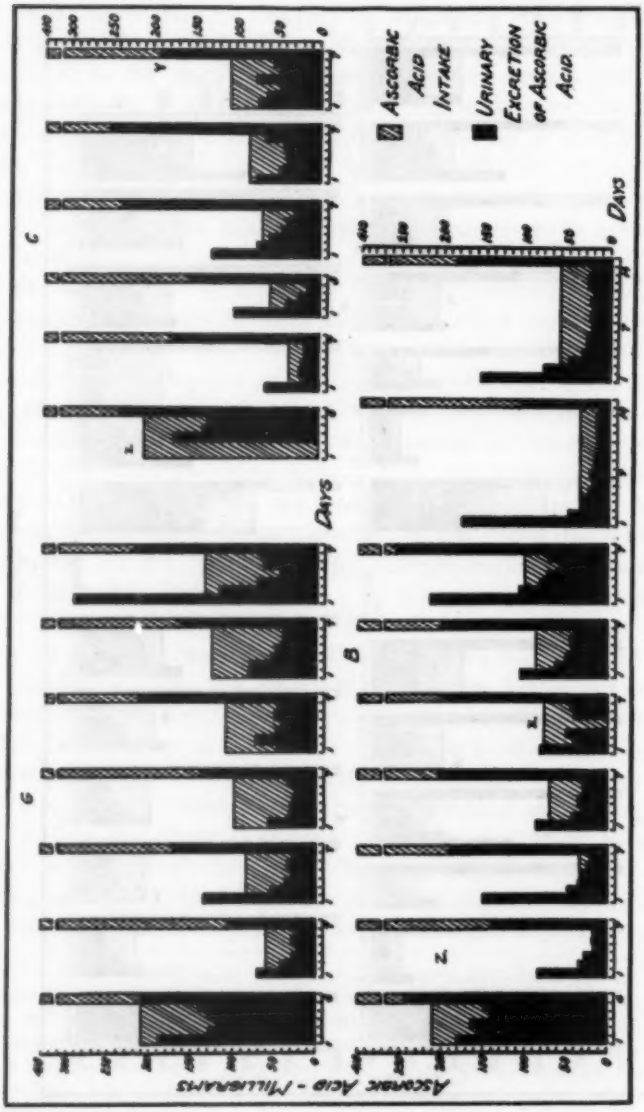
Ascorbic Acid - Milligrams
Ascorbic Acid Intake
Urinary Excretion of Ascorbic Acid
Days
A
B
C

Figure 1b

ments is very similar to King's estimate[3] of a desirable allowance for normal persons, arrived at by a different method. Standard guinea pigs are protected from gross scurvy by an intake of 0.5 mg. of ascorbic acid per day, but twice this amount is required for normal tooth structures if no other stress is brought to bear. Larger intakes are necessary to permit good protection against injury produced by bacterial toxins (Swanson, Sigal and King, '36). If toxin injections are made at the 1 mg. level, animals show marked injury to the tooth structure. In contrast, animals receiving 3 mg. per day with the same amount of toxin, show practically no tooth lesions. In human beings, such data as are available indicate that about 15 to 25 mg. per day are essential barely to protect from scurvy. If the requirement for tooth growth in human beings is analogous to that of guinea pigs, 50 mg. would permit the normal development of teeth if no unusual stress occurs. To provide good protection against the injury produced by infection or bacterial toxin, an intake of 80 to 100 mg. per day would be required. On this basis, King expressed the opinion that 100 mg. of vitamin C per day is a desirable allowance for normal persons of all ages, with the exception that this allowance should be increased by 50 mg. for pregnant and lactating women.

On the basis of the present studies, one may assume that such an allowance would permit a relatively high degree of tissue saturation in normal adults under ordinary conditions.

It must be borne in mind that these experiments involved vitamin C supplements as synthetic ascorbic acid, in addition to a basal diet which was distinctly acid forming. The median urinary pH for the various subjects was as follows: for F 5.9, for D and G 5.6, for B 5.5, for A 5.4, for C 5.3 and for E 5.0. In five of the seven cases, the mean, median and theoretical mode were the same. The effect of the reaction of the diet on excretion, storage and requiremnt of vitamin C is still unsettled (Hawley et al., '36; Goddard and Preston,

[3] King, C. G. 1938 Paper given before Am. Home Econ. Assoc., Pittsburgh, and personal communication.

'38). Preliminary experiments in this laboratory [4] indicate that there are individual differences in the effect of potential acidity or alkalinity of the diet on urinary excretion of vitamin C. This is in accord with the observations of Goddard and Preston ('38).

Factors affecting urinary excretion of vitamin C

Exercise. Although these experiments were not designed to study the influence of such conditions as exercise and environmental temperature upon the apparent requirement for vitamin C, some incidental observations of this sort have been made. For example, following two periods in which the the daily intake of vitamin C was 110 mg. subject G excreted 216 and 237 mg. when a 400 mg. test dose was given. During a third period with a similar intake, the subject played base-

TABLE 3

Effect of environmental temperature on the urinary excretion of vitamin C

DATES	TEMPERATURE (°F.)			DAY OF EXPERIMENT						RESPONSE TO TEST DOSE
	Mean minimum	Mean maximum	Mean	1	2	3	4	5	6	
				mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Aug. 5–11	67	86	77	119	71	46	52	32	31	182
Sept. 11–17	49	65	55	161	84	70	76	63	61	231

ball frequently. The response to the test dose following this period was only 156 mg. Since this subject's behavior with respect to vitamin C output was somewhat more variable than that of the other subjects studied, one cannot say with certainty that this variation was due to unusual exercise. However, increased use or disappearance of ascorbic acid following violent exercise has been reported (Hamel, '37).

High environmental temperatures. Since the results obtained during the period from October to June could not be duplicated during a hot period in August, it is believed that high environmental temperatures affect the urinary excretion of vitamin C. The figures for urinary output of vitamin C obtained with the same subject during two periods when the daily intake was 100 mg. will serve to illustrate (table 3).

[4] Unpublished data.

In this connection, the excretion of vitamin C in sweat has been noted by Lilienfeld, Wright and MacLenathen ('36) and Bernstein ('37). Further studies by Wright and MacLenathen [5] have resulted in the conclusion that while vitamin C is excreted in the sweat, it is doubtful that large amounts of this substance are lost in that manner. Martini and Torda ('37) have demonstrated that high temperatures decrease the ascorbic acid content of guinea pig tissue. Whether the lower urinary excretion during the period when the environmental temperature was high is due merely to a relatively larger excretion through the skin, or whether it is indicative of a true lowering of tissue reserves is not clear. Whatever the cause, the results obtained during hot weather differed from those obtained during the fall, winter and spring when the indoor temperature was relatively constant.

Drugs. The subjects were instructed to refrain from the use of drugs during the experiment, since the effect on vitamin C metabolism of certain preparations which are rather commonly used is either not known or in dispute. However, in the course of these experiments, a physician prescribed a medication containing atropine for subject A, and she took it during 1 week of the experiment. The daily excretion of vitamin C was greater when atropine was taken, as compared with similar periods preceding and following the use of the drug, but the response to the test dose was much lower, indicating that the subject was less well saturated. Wright [5] has observed a similar reaction when atropine was administered.

SUMMARY

A method is described for estimating the minimum intake of ascorbic acid which will just maintain the tissues in a state of complete saturation.

Of seven subjects whose requirements have been studied by this method two required between 70 and 85 mg. of ascorbic acid to maintain tissue saturation, three required between 85

[5] Wright, I. S. Personal communication.

and 100 mg. and two required more than 100 mg. of ascorbic acid per day.

On the basis of body weight, the range in requirement to maintain complete saturation for these subjects was from about 1 to 1.6 mg. per kilogram per day.

Incidental observations on the pH of the urine, the effects of exercise, high environmental temperature and atropine are recorded.

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THE ROLE OF RIBOFLAVIN AND OTHER FACTORS OF THE VITAMIN-B COMPLEX IN THE NUTRITION OF THE PIG [1]

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TWO FIGURES

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INTRODUCTION

In a previous publication Hughes ('38) demonstrated that thiamin, riboflavin, nicotinic acid and the filtrate factor (chick anti-dermatitis vitamin or factor 2) were important in the nutrition in the pig. The studies were continued to ascertain more definitely the role of riboflavin, thiamin, the filtrate factor and factor 1 (rat anti-dermatitis vitamin or vitamin B_6) in the growing pig.

EXPERIMENTAL

The conditions of this experiment were similar to those already reported by Hughes ('38). The same, or similar, salt mixtures, cod liver oil, casein, rice screenings, rice bran filtrate, nicotinic acid, thiamin and whey adsorbate were used. Wherever possible pure vitamins were given; where this was impossible vitamin concentrates were employed. The rice screenings used were medium sized pieces of commercial polished rice thoroughly cleaned at the mill. Its content of riboflavin, nicotinic acid and factors 1 and 2 is not known (factor 1, the rat anti-dermatitis factor or vitamin B_6; factor 2, the chick anti-dermatitis factor or filtrate factor); however, all

[1] Aided by a grant from American Dry Milk Institute, Inc.

grains that have been tested are low in riboflavin and the pellagra-preventive factor (Almquist, Jukes and Newlon, '38; Sebrell, '34).

The protein content of the diets was about 13.75%. Cod liver oil was given each week, 5 cc. until the pigs weighed about 70 pounds, and 10 cc. thereafter. The thiamin and nicotinic acid were given in solution, by mouth, twice each week.

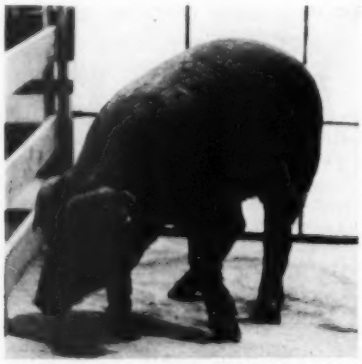

Fig. 1 Showing crippled condition of a pig in lot 2 on a diet deficient in riboflavin. Note the affected ankles, the spreading toes, the bent knees and abnormal positions of the feet.

RESULTS

Except for one pig, which gained very slowly, those in lot 1 (deficient in factors 1 and 2) grew at a moderate rate. Early in the experiment they chewed the sides of the pen and one pig developed an abnormal walk (goose-stepped). The hair and skins of the pigs were normal except for the one that gained very slowly. This pig seemed normal, but the hair was dry. At the close of the experiment a rice-bran filtrate containing largely the filtrate factor and some factor 1 was given, which resulted in an immediate increase in appetite, rate of gain and physical well being.

Those pigs fed a diet deficient in riboflavin (lot 2) gained very slowly, defecated frequently semi-fluid fecal matter, became crippled (see fig. 1) and walked with difficulty. Two of them become bowed in their front legs, turned out at the ankles, stood extremely close at the hocks and the ankles of the hind legs were bent forward excessively. They gained at the rate of 0.64 pound per day; whereas pigs in lot 5, fed the same except their diet contained riboflavin, gained 1.53 pounds per head daily. Three of them were placed in a separate pen

TABLE 1

Diets fed various groups

FEEDS, CONCENTRATES AND VITAMINS	LOT NUMBERS				
	1	2	3	4	5
Rice screenings, pounds	87	83	83	82	82
Casein, purified, pounds	..	9	9	..	..
Casein, commercial, pounds	9	..	..	9	9
Whey adsorbate, pounds	1	..	1	1	1
Rice bran filtrate,[2] pounds	..	5	5	5	5
Salt mixture, pounds	3	3	3	3	3
Nicotinic acid	14.7 mg.[1]	16.5 mg.[1]	14.0 mg.	..	14.1 mg.
Thiamin	3.7 mg.	4.0 mg.	..	3.5 mg.	3.5 mg.

[1] Milligrams per hundred weight per day.

[2] Fifteen grams of rice bran were utilized in producing 1 cc. of the rice-bran filtrate (Jukes, '37). In diets 2, 3, 4 and 5, 5 pounds of rice-bran filtrate were mixed with the other feeds. The rice-bran filtrate, therefore, represented 5% of the diet.

after being on the experiment for 72 days and were fed riboflavin in the form of whey adsorbate (1% of the diet) for 21 days. This resulted in improved well being and a slight increase in rate of growth, but did not result in any noticeable improvement in their walking ability.

The pigs in lot 3 (tables 1 and 2) were fed a diet deficient in thiamin. Some thiamin was furnished by the rice, whey adsorbate and the rice-bran filtrate. The pigs ate well for a time, then their appetites decreased. The outward appearance of these hogs was moderately good except that some of them showed a tendency toward leg weakness. Two pigs in

this lot died suddenly after being in the experiment 63 and 67 days, respectively, and at autopsy showed very few lesions. One of them showed a cystic bladder with excessively thickened walls. The other pig had a very small spleen. Except for these abnormalities the other organs seemed normal. There was no evidence of pneumonia, of flabby hearts, of abnormal livers or kidneys, and the digestive tract presented

TABLE 2

Average daily gain, feed consumed for 100 pounds of gain and other data for the various groups

Lot number	*Number of pigs*	*Average daily gain in pounds*	*Feed consumed for 100 pounds of gain, in pounds*	*Number of deaths*	*Autopsy and other findings*
1	5	0.83	324	..	Pigs scoured some, one pig gained very slowly, was very unthrifty with watery eyes and dry hair and skin.
2[1]	5	0.64	340	..	They soon showed lack of appetite, all somewhat crippled, scoured some throughout the experiment.
3	5	1.16	350	2	At autopsy one showed a cystic bladder, the other appeared normal except that the spleen was very small.
4	5	1.70	288	..	Scoured some early in test, otherwise normal.
5	5	1.53	293	..	One pig breathed heavily, otherwise normal.

[1] Three pigs were given whey adsorbate after they had been on the experiment 72 days—while they became more thrifty and their physical well being was improved they did not recover from their crippled condition. For the average beginning and final weights see figure 2.

no evidence of gastritis, hemorrhage or enteritis. Crystalline B_1 was given as a curative measure to three that remained at the close of the experiment, which resulted in increased appetites and they became definitely more active.

Lots 4 and 5 are of particular interest because those in lot 4 gained faster than those in lot 5. The diet fed lot 4 was not

supplemented with nicotinic acid; however, there was apparently enough in the rice-bran filtrate to satisfy their needs. Rice-bran filtrate has been shown by Sebrell and co-workers ('38) to supply the pellagra-preventive (anti-blacktongue) factor. When nicotinic acid was given to the pigs in group 5 they did not gain as fast as those in group 4, which may have been due to an excess of nicotinic acid in this particular diet.

DISCUSSION

The most striking result of the present investigation was the slow growth and the crippled condition of the pigs in lot 2 fed a diet deficient in riboflavin. When riboflavin was added as a curative measure there was an improvement in the physical well being of the animals but they did not recover from their crippled condition, which suggests that permanent injury had taken place due probably to nerve degeneration (Phillips and Engel, '38; Wintrobe, Mitchell and Kolb, '38). That the crippled condition was not some form of rickets was shown by the fact that one animal was killed and showed no evidence of it at autopsy. The bone ash of the humerus was 65%. Riboflavin has thus been shown to be necessary in the nutrition of the pig.

That thiamin is necessary for the pig was shown previously (Hughes, '38). This observation was confirmed by the fact that relatively slow growth was exhibited by pigs in group 3 fed a diet deficient in this factor (fig. 2) as contrasted with lot 5, and that two of the five pigs in this lot died after being on the experiment about 9 weeks. While some of the pigs showed a slight weakness in their legs, they did not exhibit evidence of beriberi paralysis as reported by Mollgaard ('38). The addition of crystalline B_1 to the diet of the three pigs remaining at the close of the experiment resulted in increased appetites, a more thrifty appearance and they became definitely more active.

Those pigs fed a diet deficient in the filtrate factor (factor 2) and factor 1 (vitamin B_6) (lot 1) did not grow normally.

They chewed the sides of their pen, walked poorly and appeared thinner than pigs in lots 4 and 5. Several became crooked in their hocks and one 'goose-stepped' with his hind legs. The addition of rice-brain filtrate (filtrate factor and factor 1) at the close of the experiment caused an immediate

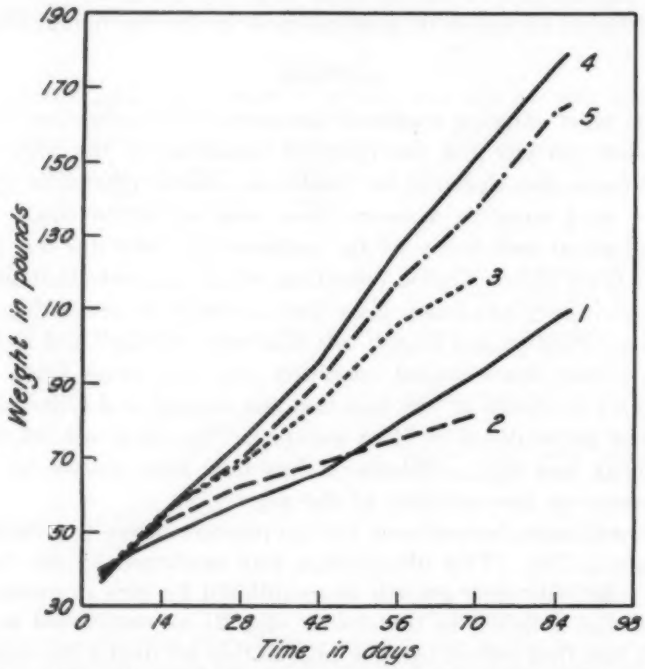

Fig. 2 Growth curves of the various lots. The figures on the graph refer to the lot numbers. The average age of the pigs in the various groups at the beginning of the experiment was 72 days.

increase in appetite and physical well being. Unfortunately we were dealing here with a double deficiency (factor 1 and factor 2).

The rate of gain of those in lot 4 was exceptionally good. Their growth rate and physical well being can be attributed to the fact that they were receiving a complete diet; that the

rice-bran filtrate fed at a 5% level was supplying the needs of the pigs for the filtrate factor, vitamin B_6 and nicotinic acid. The rate of gain (fig. 2) of the pigs in lot 5 was less than that for lot 4. Apparently the addition of about 14 mg. of nicotinic acid per hundred weight each day to the pigs in addition to that received in the rice-bran filtrate in some way slightly depressed their growth. The food utilization of both groups was excellent. It was superior to that of lots 1, 2 and 3.

SUMMARY

Using a purified diet it has been shown that riboflavin is necessary for normal growth and physical well being in the pig. Further evidence is presented showing that thiamin too is necessary for the proper nutrition of the pig.

Feeding a diet without rice-bran filtrate (containing factors 1 and 2) resulted in slow growth, which suggests that one or both of these factors are necessary in the nutrition of the pig.

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DETERMINATION OF A CURVE OF RESPONSE TO SYNTHETIC CRYSTALLINE THIAMIN, FOR USE IN THE VITAMIN B_1 ASSAY OF FOODS BY THE RAT-GROWTH METHOD [1]

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TWO FIGURES

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Chemical methods for the determination of vitamin B_1, or thiamin, in foods have not been perfected, and suggested procedures for extracting the vitamin from foods before application of chemical methods are not entirely satisfactory (Williams, '38).

Until suitable chemical methods are devised, it will be necessary to continue the determination of vitamin B_1 in natural foodstuffs by means of a biological method. The accuracy of biological determinations, using either rats or pigeons, may be increased by the use of curves of reference or curves of response, as recommended by Daniel and Munsell ('37) and by Coward ('38). Coward has published such curves for pigeons and for rats using the international standard of reference, an adsorption product on fullers' earth of an extract of rice polishings. There have been a number of reports on the use of the crystalline vitamin B_1 (Heyroth, '36; Kinnersley et al., '35; Waterman and Ammerman, '35), but no work has been reported using a large number of animals fed varying levels of thiamin and giving the resulting curve of response. The work here reported may be of interest to other workers using similar methods and synthetic crystalline thiamin as their standard of reference.

[1] Published with the approval of the director of the Hawaii Agricultural Experiment Station.

METHODS

The mothers of the experimental rats were fed the following diets during the breeding and lactation periods: whole-milk powder, 400 gm. (or 300 gm. whole-milk powder and 100 gm. skim-milk powder); whole-wheat flour, 1,025 gm.; wheat germ, 40 gm.; salt mixture,[2] 10 gm.; sodium chloride, 10 gm.; soybean meal, 100 gm. One-half of the mothers received the diet containing only whole-milk powder; the other half received the diet containing whole-milk powder and skim-milk powder. On or before the seventh day all litters were reduced to eight or nine young. Records of the weights of the young were made at birth, 7, 10, 14 and 21 days. No differences in weights of rats at birth or at weaning were noted between the litters raised on the two diets. Neither were the reactions to the experimental diet and procedure different for the rats from the mothers fed the two breeding diets.

At 21 days of age, the rats used in the thiamin tests were weaned and placed on the Chase and Sherman diet: vitamin B-free casein, 18%; salt mixture, 4%; cod liver oil, 2%; butterfat, 8%; cornstarch, 53%; autoclaved yeast, 15%. Rats were kept in individual cages with ½ inch mesh wire screens above the bottom of the cages to reduce consumption of excreta. The rats were weighed weekly for the first 2 weeks and daily after that. When the rats had maintained the same weight or lost 1 to 3 gm. each day for 3 or 4 days, the feeding of the thiamin supplements was started and continued for a period of 5 weeks.

Synthetic crystalline thiamin [3] was kept in a thoroughly dry condition by placing it in a brown glass bottle over calcium chloride except for the short period during the weighing process. A 10% alcoholic solution, containing 10 μg. of thiamin in 0.1 ml., was found to be most satisfactory. A 20% alcoholic solution was used for preliminary experiments, but it was believed that some evaporation took place and that

[2] Nine parts calcium carbonate and 1 part ferric citrate.
[3] Purchased from the Winthrop Chemical Company.

greater accuracy could be obtained by using a 10% solution. A fresh solution made each week was placed in a brown glass bottle and kept in a refrigerator, only the small portion needed for each day's feedings being removed at one time. This solution was fed from calibrated pipettes directly into the mouths of the experimental animals. The rats took the solution avidly so that after the first or second feeding there was no danger of losing even the smallest drop.

To supply quantities of thiamin equivalent to 1, 2, 3 and 4 μg. daily (except Sunday), the necessary volumes of the solution were fed three times per week. For 8 and 16 μg. daily (except Sunday), the solution was fed six times per week.

An even distribution of the sexes in each group of rats was desired. Unfortunately, due to mistaken sex identification at the beginning of the experiment, it was necessary to discard some of the rats in several groups even though they responded satisfactorily, in order to equalize the sexes. All the groups receiving 2 to 16 μg. per rat daily had equal division of sexes although the number of pairs in each group varied. In the 1-μg. group there were thirteen females and twelve males, and in the negative control group there were more males than females. The records of 192 rats were used in tables 1 to 4 and figures 1 and 2.

RESULTS

The average weights of the seven groups of rats when placed on the thiamin-free diet at 21 days were very uniform. The mean weight for the 192 experimental rats was 41.1 ± 0.33 gm. with a standard deviation of 4.5 ± 0.23 gm. The rats were depleted of their stores of vitamin B_1 in about 20 days. Animals that did not deplete within 24 to 25 days were found to be unsuitable for experiments, and it was necessary to discard them. When such rats were given supplements, their gains varied greatly from that of the remainder of the group. When they were maintained on the basal diet only, refection apparently took place as their weights in-

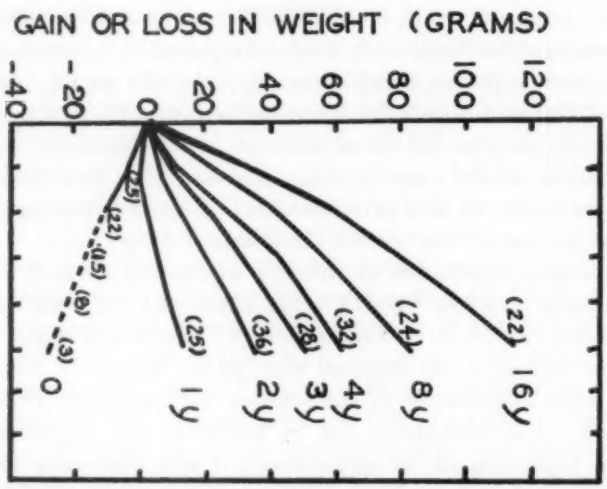

Fig. 1 Average growth response of groups of rats fed varying quantities of synthetic vitamin B_1 (thiamin) after depletion of vitamin B_1 stores. The figures in parentheses show the number of rats used for each test. The broken line indicates the occurrence of the first death in the control group. Spaces on abscissa indicate weeks.

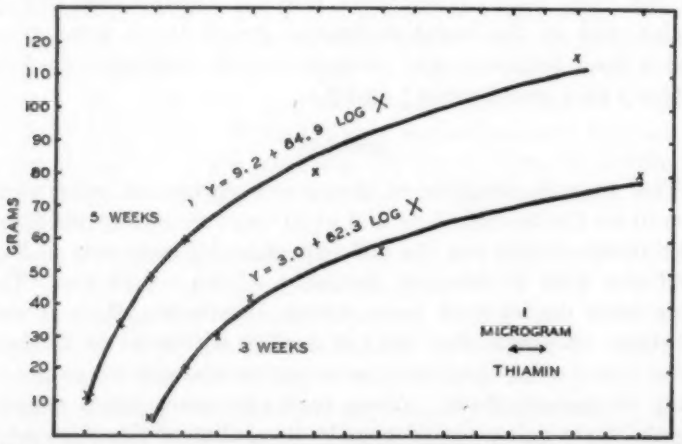

Fig. 2 Calculated curves of response relating gains in weight of rats after 3 weeks and after 5 weeks to doses (in micrograms) of synthetic crystalline thiamin. The crosses indicate actual mean gains in weight of groups of rats fed daily doses of 1, 2, 3, 4, 8 and 16 μg. of thiamin.

creased, even though they previously showed evidence of depletion.

In table 1 are summarized data on the number of rats in each supplement group, the average time for depletion of each group, the average weights of each group at different periods of the experiment and the mean gains in weight of rats in each group after 3 weeks and after 5 weeks of supplement feeding.

The standard errors and 't' values of the mean gains in weight after 3 and 5 weeks of supplement feeding appear in

TABLE 1

Summary of the results of feeding rats a thiamin-free diet and graded doses of synthetic crystalline thiamin for 3 weeks and for 5 weeks

SUPPLEMENT OF THIAMIN	NUMBER OF ANIMALS	AVERAGE WEIGHTS		AVERAGE DEPLETION PERIOD	FINAL AVERAGE WEIGHTS		MEAN GAINS	
		At weaning	At depletion		At 3 weeks	At 5 weeks	In 3 weeks	In 5 weeks
μg.		*gm.*	*gm.*	*days*	*gm.*	*gm.*	*gm.*	*gm.*
0	25	41	70	21				
1	25	42	71	20	76	83	5	12
2	36	41	70	20	90	104	20	34
3	28	40	70	20	101	118	31	48
4	32	41	70	20	112	130	42	60
8	24	41	69	20	126	150	57	81
16	22	42	68	20	148	184	80	116

table 2 (Fisher, '36). With the exception of the group of rats receiving the 1 μg. thiamin supplement for 3 weeks, all mean gains in weight for both periods are highly significant as indicated by the 't' values.

The differences between the mean gains of the groups of rats receiving the different thiamin supplements, the standard errors of the differences and 't' values are presented in table 3. All differences in mean gains are statistically significant. Further, it is apparent that the differences in mean gains can be just as accurately ascertained at the end of 3 weeks of supplement feeding as at 5 weeks. The savings in time and cost for a 3-week feeding period in comparison with a 5-week

feeding period are obvious. Coward ('38) has pointed out that for her rat colony a 3-week feeding period was satisfactory for vitamin B_1 tests and has shown that a test carried out in 2 weeks was almost as accurate as one continued for 3 weeks ('36).

TABLE 2

The standard errors and 't' values of the mean gains in weight of six groups of rats after 3 weeks and after 5 weeks of supplemental feeding of synthetic crystalline thiamin

THIAMIN	NUMBER OF RATS	3 WEEKS			5 WEEKS		
		Mean gain	Standard error of mean	't' [1] value	Mean gain	Standard error of mean	't' value
µg.		*gm.*	*gm.*		*gm.*	*gm.*	
1	25	5	2.1	2.4	12	3.0	4.0
2	36	20	1.4	14.3	34	1.9	17.9
3	28	31	2.3	13.5	48	2.6	18.5
4	32	42	1.6	26.2	60	2.5	24.0
8	24	57	2.6	21.9	81	3.4	23.8
16	22	80	3.7	21.6	116	5.4	21.5

[1] For samples of n larger than 19, a 't' value greater than 2.1 indicates significance at the 5% probability level, and a 't' value greater than 2.9 indicates significance at the 1% probability level (Fisher, '36, table IV).

TABLE 3

The standard errors and 't' values of differences between the weight gains of groups of rats fed supplements of synthetic crystalline thiamin

INTERVAL OF THIAMIN SUPPLEMENT	3 WEEKS			5 WEEKS		
	Difference	Standard error	't' value	Difference	Standard error	't' value
	gm.	*gm.*		*gm.*	*gm.*	
1–2	15	2.4	6.2	22	3.3	6.7
2–3	11	2.5	4.4	14	3.1	4.5
3–4	11	2.7	4.1	12	3.6	3.3
4–8	15	2.9	5.2	21	4.1	5.1
8–16	23	4.4	5.2	35	6.2	5.6

In the assay of natural foods for thiamin by the biological method, the minimum number of experimental rats that must be used to obtain reliable results is also extremely important. It is apparent from the 't' values given in table 3 that a mean difference only half as large as that found would be

statistically significant, other factors being maintained, or that the difference found would have been satisfactory if less than half the number of rats in each treatment had been used.

In order to obtain an accurate estimation of the error variance of gain in weight of individual rats at different periods after supplemental feeding of thiamin, twenty-one rats from each group or treatment were selected at random and the methods for the analysis of variance of an experiment having twenty-one replications and six treatments were applied, separate analyses being made for the 3-week and 5-week periods. The results are presented in table 4. From

TABLE 4

Analysis of variance of gain in weight of twenty-one rats in each of six thiamin treatments at 3 weeks and at 5 weeks

SOURCE OF VARIANCE	DEGREES OF FREEDOM	3 WEEKS VARIANCE	5 WEEKS VARIANCE
Treatment	5	14,790	27,173
Replication	20	82	168
Error	100	155	311
Total	125	..	..
Standard error, gain in weight of a single rat		12.4	17.6

the total variance of the gain in weight of the 126 rats measured at each period the variances due to treatment and replication have been subtracted leaving variance or mean square deviation for experimental error. The square root of the error variance yields the standard error of a single observation; the standard errors are 12.4 gm. and 17.6 gm. for gain in weight per rat after 3 weeks and after 5 weeks respectively. If a difference of 11 gm. gain in weight between two treatments at 3 weeks is considered satisfactory, it is then a simple calculation to determine the number of rats required in each treatment to demonstrate the statistical

significance of this difference.[4] About twelve and fifteen rats, respectively, would be required to demonstrate the significance of differences of 11 gm. and of 14 gm. for the 3- and for the 5-week periods. From this standpoint the 3-week period is not only more economical in time and labor but also in number of rats required to yield statistically significant differences. Larger differences such as are found between 1- and 3- or between 2- and 4-μg. thiamin supplements can, of course, be proved with smaller numbers.

The curves of response for 3 weeks and for 5 weeks have been calculated according to the method of Coward ('38, fig. 2). The equation of the curve is $y = \bar{y} + b \log X$, where

y = gain in weight in grams during feeding period
$\bar{y}$ = mean value of y
X = daily dose of thiamin in micrograms
x = log of daily dose of thiamin in micrograms
$\bar{x}$ = mean value of x
b = regression coefficient, or $\frac{\Sigma y\,(x-\bar{x})}{\Sigma\,(x-\bar{x})^2}$

The actual mean gains in weight, especially in that portion of the curve in which the curvature is greatest, fall very close to the calculated curves for the gains in weight. Experiments which result in gains in weight falling in this portion of the curve are, therefore, the most sensitive.

The use of a curve of response in assaying foods of unknown vitamin content has been fully discussed by Coward ('38).

[4] Calculation

Standard error of mean $\frac{12.4}{\sqrt{n}}$

Standard error of difference between two means $\frac{12.4 \times \sqrt{2}}{\sqrt{n}}$

't' value required for minimum level of significance when $n = 10$ more or less is approximately 2.2. $\frac{12.4 \times \sqrt{2} \times 2.2}{\sqrt{n}}$

Assuming that a minimum difference of 11 gm. is as great as may be conveniently proved. $\frac{12.4 \times \sqrt{2} \times 2.2}{\sqrt{n}} = 11$

Solution $n = 12$

SUMMARY AND CONCLUSIONS

1. To determine the growth response of rats to graded doses of thiamin, 192 rats were given a thiamin-free diet at 21 days of age; at depletion of thiamin stores after about 20 days, twenty-five rats were used as controls, and 167 rats were fed daily doses of synthetic crystalline thiamin at six levels (1, 2, 3, 4, 8 and 16 μg.) for a period of 5 weeks. The results show a definite curvilinear relationship between the mean gains in weight after 3 weeks and after 5 weeks and the quantity of thiamin administered; $y = 3.0 + 62.3 \log X$ for 3 weeks, and $y = 9.2 + 84.9 \log X$ for 5 weeks.

2. A statistical analysis of the results of a 5-week feeding period and those of a 3-week feeding period shows that the shorter period gives as significant results as the longer period.

3. The number of rats necessary for a significant biological assay of foods for their thiamin content for a particular colony has been determined to be twelve.

4. When supplements providing quantities of thiamin between 1 and 4 μg. are fed to groups of twelve rats six times a week, the responses should be sufficiently accurate to distinguish differences of 1 μg.

5. Curves of response relating the doses of thiamin to gains in weight are given for the 3-week and the 5-week feeding periods.

The author is indebted to Prof. E. A. Hoy of the Mathematics Department, University of Hawaii, and to Dr. Lyman Dean, Chemist, Hawaii Agricultural Experiment Station, for criticism of the manuscript and for assistance with the statistical analysis.

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PRODUCTION OF GOITER IN RATS WITH RAW AND WITH TREATED SOY BEAN FLOUR

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Recent reviews indicate that endemic goiter can be controlled, except in certain areas of New Zealand, by administration of iodine. However, discrepancies in the data furnished by surveys leave considerable doubt that iodine deficiency per se is the primary cause of goiter. Numerous surveys have shown that iodine intake is low in goiter areas but is not lower than the intake in many goiter-free areas. The nature of endemic goiter has been reviewed recently by McCarrison ('37) and by Hellwig ('37). They both agree that there are two types of endemic goiter. In mountainous areas it occurs as a diffuse parenchymatous goiter in childhood and as adeno-parenchymatous nodular goiter in adults with its degenerative forms. The characteristic type in plains areas is a diffuse colloid goiter. A complete explanation of these differences probably depends on more than a simple iodine deficiency.

With the exception of studies on goiter in rabbits (Webster and Chesney, '30) most recent experiments have been made upon rats. The rachitogenic diet of Steenbock and Black or modifications of it, is the only diet reported which has consistently produced goiter in rats in different laboratories. Levine, Remington and von Kolnitz ('33) made a critical study of the goiter obtained with this diet. Hellwig ('35) was able to produce goiter in rats by feeding rolled oats, cornmeal, distilled water and calcium but the iodine content

of the diet was not determined. Under certain experimental conditions McCarrison ('33) found that some native diets of India will produce goiter in rats. Diets of highly purified materials even though they are low in iodine have uniformly failed to produce enlarged thyroids in rats.

In order to study some of the dietary factors which may influence the thyroid gland, studies were started with a purified diet low in iodine (Carpenter and Sharpless, '37). With the rats used, satisfactory goiter was obtained neither with this diet nor with the diet of Levine, Remington and von Kolnitz ('33). The addition of substances which have been reported to increase the need for iodine (such as unsaturated fats, calcium, chlorides, fatty acids and various nitriles) did not produce a marked degree of enlargement. Since McCarrison ('34) had reported that soy beans would produce a goiter in rats which was not prevented by iodine, a diet composed mostly of soy bean flour was fed and a marked thyroid enlargement was obtained (Sharpless, '38).

Soy beans have a high nutritive value (Horvath, '38) and probably provide as complete a diet as can be obtained from any single vegetable source. The protein is adequate for promoting normal growth and is 'physiologically good.' The content of vitamins A, B complex and E is adequate and it has a high mineral content, especially of calcium and phosphorus.

EXPERIMENTAL

Rats, both male and female, weighing 60 to 80 gm.; were used for all of the studies. They were bred on an adequate stock ration which has been described elsewhere (Carpenter and Sharpless, '37). It was found that animals which weighed more than 60 gm. adjusted themselves to the diet more readily than smaller animals. Wire cages with wire mesh bottoms were used. They were washed frequently. These rats were kept in a room with only a few other experimental animals. Those on diets which had some iodine added were fed in a separate room. The soy bean flour was unprocessed and was

prepared from Michigan-grown beans. Precautions were taken to insure fresh flour and avoid rancidity. Different samples obtained over a period of 4 years have varied from 15 to 20% in ether extractable material but have been uniformly satisfactory in producing goiter.

Formulae of the diets studied are shown in table 1. Rats fed the first diet (no. 62) which contained 93% soy bean flour did not do well. For the first few days they had diarrhea, were bloated and about 50% of them died. Those that survived developed a tolerance for the diet and grew to maturity although they never had a healthy appearance. When 5% of

TABLE 1

Formulae of soy-bean goiterogenic diets

	DIET NO.							
	62	62 A	143	153	168	203	204	213
Unprocessed soy bean flour %	93.0	75.0	75.0	75.0	75.0	75.0	75.0	35.0
Calcium carbonate %	3.0	3.0	0.5	0.5	0.5	0.5	0.5	0.5
Sodium chloride %	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Butterfat %	0.0	5.0	5.0	5.0	5.0	5.0	0.0	5.0
Yeast %	3.0	3.0	3.0	3.0	3.0	0.0	3.0	3.0
Sucrose %	0.0	13.0	15.5	15.5	15.5	18.5	20.5	55.0
Viosterol—15 drops per kilogram food	—	—	—	—	+	—	—	—
Iodine as K I	0.0	0.0	5 mg. per kg.	0.0	0.0	0.0	0.0	0.0

butterfat and 13% sugar displaced 18% soy bean flour (diet 62 A), all of the rats ate the diet and grew at approximately 40% of the normal rate. Because of the numerous reports on the goiterogenic effect of calcium, 3% of calcium carbonate was fed. In later experiments 0.5% of calcium carbonate was used (diet 153), and goiters of the same magnitude were obtained. Rats fed this diet grew at about 60% of the normal rate. With some exceptions they attained normal adult size, reproduced and in general appeared normal. However, if the diet was old or rancid, they did not grow so well and they lost much of their hair. Although the desirability of improvement was recognized this diet was considered satisfactory for further study.

In order to determine whether the supplements were of value, the yeast was omitted from diet 203 and the butterfat was omitted from diet 204. Goiters developed with both diets but growth was not so good as when both supplements were present. To be sure that a rachitic condition was not contributing to the development of goiter, viosterol was added to one diet (diet 168). Viosterol had no apparent effect on the growth or size of goiter. Iodine was added to one diet (143) to determine whether the goiterogenic property was active in the presence of iodine. Finally the soy bean flour content was reduced to 35% and sugar substituted (diet 213). There is less iodine in sugar than in soy bean flour and therefore this change effected a decrease in the iodine content of the diet. If there is a direct relationship between iodine deficiency and goiterogenic activity, this diet should be more goiterogenic than any of the others.

Iodine was determined in the dried thyroid glands by the method of Trevorrow and Fashena ('35). In the diet oxidation was carried out in an atmosphere of oxygen and the oxidation products were collected in a dichromate-sulfuric acid mixture. The determination was then completed by the Trevorrow and Fashena method. When freshly purified chemicals were used and care observed in each step of the procedure the method gave satisfactory results.

RESULTS

The degree of thyroid enlargement and the iodine content of the glands are shown in table 2. To avoid confusion only results on the normal, 75% soy bean flour, 35% soy bean flour and iodine containing soy bean flour diets are recorded. There was no significant difference between goiters obtained with the other diets and those shown for diet 153.

The average thyroid size of 130 rats fed the goiterogenic diet for 7 weeks was approximately four times normal. Some rats developed larger thyroids if fed the diet longer but the average of a number of rats fed for 160 days was about the same as that shown for 49 days. As an exception, a thyroid

nine times normal size and weighing 230 mg. has been obtained in a rat fed for 9 months. A period shorter than 7 weeks has not been satisfactory. Seven rats in table 2 show that goiters were not well developed in 4 weeks. For these reasons all of the studies were based on an experimental period of 7 weeks.

Stock animals had a thyroid iodine content of 4.2 μg. which was 0.1% of the dry weight. This is within normal limits but it approaches the value at which histological changes might be expected. Therefore it will be called the minimum

TABLE 2

Production of goiter with soy bean flour and prevention with iodine

DIET	IODINE CONTENT OF DIET	TIME ON EXPERIMENT	NUMBER OF RATS	AVERAGE THYROID WEIGHT		IODINE CONTENT	
				Total	Per 100 gm. Body weight	Average Total	Dry weight Basis
	μg/kg.	*Days*		*mg.*	*mg.*	*μg.*	*%*
Stock	95		31	20.3	10 ± 0.3	4.2	0.10
153	50	49	130	57.2	40 ± 0.8	0.6	0.005
153	50	29	7	28.2	25 ± 1.0	0.3	0.009
213 (35% soy bean flour)	35	49	22	23.0	24 ± 1.8	0.4	0.008
143 (0.5 mg.% K I)	3875	49	14	16.8	11 ± 0.3	10.6	0.21

requirement in this report. The enlarged thyroids had an average total iodine content of 0.6 μg which was 0.005% of the dry weight. Thyroids of rats fed diet 153 for 4 weeks and diet 213 for 7 weeks had a similar iodine content but the glands were smaller. Rats fed diet 143 which contained added iodine had an average iodine content of 10.6 μg or 0.21% of the dry weight, showing that the thyroid of rats fed soy bean flour reacts in a normal manner to ingested iodine.

HISTOLOGY

Microscopically the glands of stock animals were normal. The follicles were lined with low cuboidal cells and were well filled with colloid. The colloid did not stain so darkly as the colloid in glands getting more iodine. The goiters had

follicles lined with high cuboidal or columnar cells showing hypertrophy and hyperplasia. In the larger goiters there was practically no colloid and the epithelium had folded into the follicles so that there was a great increase in the number of very small follicles. The first change appeared to be one of hypertrophy rather than hyperplasia. Iodine prevented this change and produced a normal gland.

It is shown that iodine prevented the goiter but it is not clearly shown that iodine deficiency alone produced the goiter. Diet no. 213 which contained 35% soy bean flour had a lower iodine content than diet 153. However, the goiters obtained with this diet were not so large as those obtained with diet 153. The difference in thyroid weight is statistically significant when glands of rats fed diet 213 are compared with the whole series fed diet 153 and also (not shown in table 2) when compared with the litter-mate controls fed diet 153. If this observation is correct, the iodine requirement of rats fed the goiter diet should be greater than normal, and there should be some means of changing the goiterogenic properties of the diet without changing its iodine content or nutritional properties. These studies were made and are shown below.

Iodine requirements of rats fed soy bean flour diet no. 153. Diet no. 153 (75% soy bean flour) was used as the basal diet and iodine in the form of potassium iodide was added. Litters were divided among all of the diets so that the stock diet is a direct control of all of the diets. Results and the diets studied are recorded in table 3.

Twenty micrograms and 40 μg. of iodine added to a kilogram of food caused a reduction in size from 36 to 15 mg. per 100 gm. body weight. As shown in the table, the glands are significantly larger than normal. One hundred micrograms of added iodine (diet 209) caused a reduction in size to 12 mg. per 100 gm. body weight which is also significantly larger than normal. Five hundred micrograms (diet 210) produced glands that weighed 11 mg. per 100 gm. of body weight which is not significantly larger than normal.

All of the diets except 210 produced glands with less than 0.1% iodine and therefore furnished less than the minimum requirement. The amount of iodine necessary to produce a gland with an iodine content of 0.1% was not determined exactly but from the figures shown it can be estimated that 50 μg. added to a kilogram of diet no. 209 would furnish approximately the minimum requirement. Which means that an iodine content of 200 μg per kilogram or approximately 50 μg per 1000 calories is the minimum amount of iodine required to produce a normal gland in rats fed the soy bean flour diet. With the figures shown it can be calculated that the amount of iodine necessary to produce an optimum iodine concentration in the thyroid (around 0.2% of the dry weight, Remington, Remington and Welch, '37) is not less than 200 μg. per 1000 calories. In a normal diet, the optimum requirement is around 80 μg. per 1000 calories.

TABLE 3

Iodine requirement of rats fed goiterogenic diet

DIET	IODINE CONTENT OF DIET	NUMBER OF RATS	AVERAGE THYROID WEIGHT		IODINE IN THYROID		SIGNIFICANCE RATIO TO STOCK ANIMALS	PROBABILITY
			Total	Per 100 gm. Body weight	Average Total	Dry weight		
	μg/kg.		*mg.*	*mg.*	*μg*	%		%
Stock	95	31	20.3	10 ± 0.3	4.17	0.101		
153	50	21	47.4	36 ± 1.4	0.48	0.006	7.4	0
223	70	6	17.0	15 ± 0.6	0.57	0.015	4.4	0
208	90	50	17.7	15 ± 0.4	1.10	0.021	6.5	0
209	150	19	13.3	12 ± 0.4	2.3	0.075	2.5	1.2
210	550	10	15.7	11 ± 0.7	6.9	0.162	0.9	39

Our stock diet contained around 25 μg of iodine per 1000 calories and produced glands very similar in size and iodine content to those reported by Levine, Remington and von Kolnitz ('33 a) in stock rats fed a similar amount of iodine. Rats fed the rachitogenic goiter diet also require a similar amount. The soy bean flour diet however requires twice as much iodine as the normal. The question arises: Is this effect produced by some property of the soy beans? Raw soy bean flour has a

flavor that is objectionable to many animals and it is not so nutritious raw as cooked (Horvath, '38). In order to obtain additional information on the nature of the goiterogenic property, the effect of heat and of fat solvents on the goiterogenic ability of soy bean flour was studied.

Effect of heat and of fat solvents on goiterogenic action of soy bean flour. The diets used in these experiments were all modifications of diet 153. Litter-mate controls were fed diet 153 in each study. In place of unprocessed soy bean flour in diet 153, soy bean flour treated by one of the follow-

TABLE 4

Effect of treatment on goiterogenic property of soy bean flour

DIET	NUMBER OF RATS	AVERAGE THYROID WEIGHT		IODENE CONTENT		SIGNIFICANCE RATIO	PROBABILITY
		Total	Per 100 gm. Body weight	Average Total	Dry weight		
		mg.	*mg.*	*μg*	%		%
169 ether extracted flour [1]	19	28.5	21 ± 0.99	1.38	0.025		
225 acetone extracted flour						5.04	0
153 littermate controls	14	52.8	39 ± 2.19	0.95	0.009		
184 autoclaved flour	24	53.8	28 ± 1.2	0.63	0.006	2.48	1.2
153 littermate controls	21	62.9	39 ± 2.7	0.35	0.003		
150 steamed flour	20	31.3	19 ± 0.99	0.79	0.009	4.6	0
153 littermate controls	20	47.7	36 ± 2.3	0.51	0.004		

[1] Results with these two diets were identical and therefore they are recorded together.

ing three methods was used: 1) extracted with ether or acetone; 2) autoclaved for 30 minutes at 20 pounds pressure; 3) steam generated from iodine-free water was passed through a 10-inch column of the flour for 30 minutes. The methods of treatment did not change the iodine content but fat extraction increased the iodine content per 1000 calories. Results of these experiments are shown in table 4.

All three methods of treating the soy bean flour caused a significant reduction in its goiterogenic activity. Ether or acetone extracted flour caused a reduction in thyroid weight

from 39 to 21 mg. per 100 gm. of body weight. The total iodine content of the glands was slightly higher than that of the controls and the per cent content was much higher. Since some increase in iodine content might be expected because the iodine content of the diet per 1000 calories was slightly higher, the higher iodine content of the gland can only be interpreted in the light of results with the other diets. Autoclaved flour reduced the average thyroid size from 39 to 28 mg. per 100 gm. body weight. This is not so significant a decrease but there is only a probability of one in a hundred that it occurs by pure chance. The average iodine content per gland was raised from 0.35 to 0.63 μg. Steamed flour caused a reduction from 36 to 19 mg. per 100 gm. body weight and also an increase in the average iodine content.

Microscopically, the glands of animals fed the treated flours showed less hyperplasia than their controls. There was considerable hypertrophy of the cells and very little colloid present.

DISCUSSION

The results show that the goiterogenic action of the diet is not wholly dependent upon its iodine content. However, the glands were larger than normal in all of the experiments. The thyroids of rats fed the treated flours were similar in size to those fed the modified rachitogenic goiter diet (Levine, Remington and von Kolnitz, '33) which contained the same amount of iodine. It is therefore possible that the iodine content is low enough to produce some thyroid enlargement.

The experiments show that the high fat content of the diet does not produce the goiter. Extraction of fat decreases the enlargement but steaming the flour has the same effect even though the fat is not extracted.

Crotti ('38) has recently reported that a fungus will produce goiter in rabbits, guinea pigs and dogs. The fungus is present in goiterogenic cabbage, water supplies in endemic goiter areas and in fresh goiter material. Steam had no effect on the fungus. Since steam significantly reduced the goitero-

genic effect of soy bean flour, it is improbable that the goiterogenic agent in soy bean flour is this fungus. Crotti also found that small amounts of iodine had little effect on the goiterogenic action of the fungus whereas small amounts of iodine prevented the goiter produced by soy bean flour.

In view of the intimate relation of the thyroid and pituitary, it is always possible that thyroid changes may be secondary to changes in the pituitary. Pituitaries were weighed in all experiments and no definite change in weight could be detected in the 7-week experimental period. Longer experimental periods produced slightly enlarged pituitaries.

Marine, Baumann, Spence and Cipra ('32) have shown that organic cyanides stimulate the thyroid by depressing the oxidative systems of the body. In preliminary studies, methyl nitrile did not produce goiter in rats even though the iodine intake was low. McCarrison ('33) also found that methyl nitrile did not produce goiter in rats even in doses far greater than the amount that could be obtained from their food. However, soy beans are relatively rich in cyanogen and yield considerable quantities of cyanide on enzyme hydrolysis. Without further study this must be considered as a possible goiterogenic agent in soy bean flour.

The functional state of the thyroid of rats fed the soy bean diet as shown by metabolism tests has been the subject of preliminary studies. While the data are too few to draw definite conclusions, there is no indication that the basal metabolic rate is lower than that of litter-mate controls of the same body weight. The basal rate is consistently a little higher but probably not more than the experimental error expected with the method.

SUMMARY

1. A diet composed of unprocessed soy bean flour, yeast, sugar, butterfat, sodium chloride and calcium carbonate, when fed to rats, produces thyroid enlargement of four times normal in an experimental period of 7 weeks.

2. The minimum iodine requirement of rats fed the goiterogenic diet is two times normal or approximately 50 μg. per 100 calories of food. The optimum iodine requirement is approximately 200 μg per 1000 calories which is also twice normal.

3. Unprocessed soy bean flour contains a positive goiterogenic property that is removed or destroyed by fat solvents (ether or acetone) or by steam.

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THE UTILIZATION OF THE CALCIUM IN VARIOUS GREENS [1]

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The importance of the green leafy vegetables as sources of dietary calcium has been responsible for many investigations of the availability of their calcium. The calcium of kale (Fincke and Sherman, '35) and of Chinese cabbage (Kao, Conner and Sherman, '38) was found to be nearly as well utilized by rats as that of milk. Mallon, Johnson and Darby ('33) have reported the calcium of lettuce as more efficiently used by young women than that of milk. The maintenance requirements of young women were found by Blatherwick and Long ('22) to be satisfied by the calcium of lettuce, spinach or cabbage. Fincke and Garrison ('36) have reported that the calcium of kale is considerably more available to young women than that of spinach.

Others have found the calcium of spinach poorly utilized both by dogs (McClugage and Mendel, '18) and by young rats (Fincke and Sherman, '35; Fairbanks and Mitchell, '38; Tisdall and Drake, '38). The low availability of the calcium of spinach has been attributed to its oxalate content. Sherman and Hawley ('22) have reported that children utilize the calcium of milk better than that of spinach. That spinach tends to produce a negative calcium balance when introduced into the diet of infants has been shown by Edelstein ('32). This decrease in calcium retention of infants fed spinach was not due to its fiber content (Schlutz, Morse and Oldham, '33)

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since cellulose, in the form of Celluflour, had no effect on the calcium balance. Likewise for rats Bloom ('30) has reported that though dried spinach in the diet caused less calcium storage than the ash of the same amount of spinach, the fiber content was not responsible for the decreased retention since filter paper fed with the ash had no such effect.

EXPERIMENTAL

In these experiments the procedure used was essentially that developed by Fincke and Sherman ('35) in their work on the availability of calcium in spinach and kale. It was planned to determine the utilization of the calcium of turnip greens, tendergreen, collards and New Zealand spinach, as well as of kale grown under different conditions.

The experimental animals were albino rats reared by mothers on a stock diet of one-third whole milk powder and two-thirds ground whole wheat, which is known to support normal growth and calcification. The experimental diets were patterned after the stock diet, skim milk powder and filtered butter fat being used in place of the whole milk powder. In the control diet practically all of the calcium was supplied by the skim milk; in the diets containing the greens one-half of the skim milk was replaced by enough of the green to supply an equal amount of calcium, the difference in weight being made up with the cornstarch. The animals were fed the experimental diets from 28 days to 60 days of age, a period during which the rate of growth and calcification is very rapid. The utilization of the calcium in these diets was to be measured for this period.

The greens were grown under similar conditions and harvested at approximately the same degree of maturity. After being thoroughly washed, they were dried at 60° to 65°C. and ground in a ball mill until fine enough to pass through a 40-mesh sieve. A homogeneous sample of each sufficient for the entire experimental period was prepared.

Calcium was determined gravimetrically by the modification of the McCrudden method developed by Sherman and MacLeod

('25), and phosphorus by the method of the Association of Official Agricultural Chemists. The method of Arbenz ('17) was used to determine oxalic acid.

The same lot of both skim milk powder and whole wheat was used throughout the experimental period, the respective calcium contents equalling 1.40% and 0.037%. The greens contained the following percentages of calcium: New Zealand spinach 0.03%, kale 2.30%, collards 2.33%, turnip greens 2.39%, tendergreen 3.07%. The only one of the greens found to contain oxalate was New Zealand spinach with a content of 4.8% of anhydrous oxalic acid.

TABLE 1

Composition of the diets and their calcium and phosphorus contents

	CONTROL	KALE	NEW ZEALAND SPINACH	COLLARD	TURNIP GREEN	TENDER-GREEN
Whole wheat	65	65	65	65	65	65
Butterfat	10	10	10	10	10	10
Skim milk powder	22	11	11	11	11	11
Cornstarch	3	7	—	7	8	9
Greens	—	7	14	7	6	5
NaCl	1.3	1.3	1.3	1.3	1.3	1.3
Calcium—%	0.327	0.339	0.320	0.338	0.314	0.325
Phosphorus—%	0.418	0.357	0.366	0.314	0.360	0.351

The composition and analysis of the experimental diets are shown in table 1. The calcium contents of the diets were, as was planned, practically the same; the phosphorus contents were not widely different.

On being weaned at 28 days of age, the experimental animals were placed in individual cages with food and water provided ad libitum. A careful record of food consumption was kept so that the calcium intake of each animal would be known. The distribution of the animals to the various diets was made as even as possible in respect to initial weights and litter mates. At 60 days of age the animals were killed with chloroform. The gastrointestinal tract was removed, the weight of its contents determined and then discarded. The weight of the rat minus the weight of the gastrointestinal contents

has been termed the net body weight. The body was charred over a low flame and then ashed in a muffle furnace at dull red heat. The ash was dissolved to dilute hydrochloric acid and made up to a suitable volume, aliquots of which were used for the calcium analyses.

Representative animals were killed at 28 days of age, and the percentage of calcium in their bodies determined by analysis. The average percentages of calcium found in ten males and eleven females of this age were $0.66 \pm 0.04\%$ and $0.69 \pm 0.03\%$, respectively (on the basis of live weight). These values were used to calculate the calcium present in the 28-day-old rats at the time they were placed on the experimental diets.

RESULTS AND DISCUSSION

The growth and food consumption of the animals during the experimental period are shown in table 2. Growth was greatest on the control diet and smallest on the New Zealand spinach diet. On the other diets containing greens, growth was about

TABLE 2

Growth and food consumption on the various diets

DIET	NUMBER OF RATS	INITIAL WEIGHT AT 28 DAYS	TOTAL GAIN IN WEIGHT	NET WEIGHT AT 60 DAYS	FOOD EATEN	GAIN PER GRAM OF FOOD EATEN
Males						
		gm.	*gm.*	*gm.*	*gm.*	
Control	4	55	85	130	293	0.290
Turnip green	6	54	64	108	252	0.254
Tendergreen	6	53	66	109	258	0.256
Collard	6	56	56	103	249	0.225
Kale	3	55	61	108	255	0.240
New Zealand spinach	5	53	18	60	149	0.121
Females						
Control	5	50	62	103	249	0.249
Turnip green	4	46	56	95	240	0.233
Tendergreen	4	48	51	91	233	0.215
Collard	3	46	46	85	229	0.201
Kale	6	50	42	84	235	0.174
New Zealand spinach	3	52	16	55	152	0.105

equal to, or slightly less than, that on the control diet. Besides gaining but little weight, the animals on the New Zealand spinach diet were weak and unkempt in contrast to the normal and healthy appearance of the animals on the other diets. Smaller food consumption does not entirely account for the poor growth on the New Zealand spinach diet, for the gain in weight per gram of this diet eaten was only about one-half that of the other diets.

The calcium contents of the rats at the end of the experimental period are shown in table 3 expressed both as total and as percentage of net weight. Neither of these values in themselves measures calcium utilization accurately, for they do not take into account differences due to variations in initial store or food consumption. A more exact value for measuring utilization is expressed in the calcium utilization factor, which is the ratio of the calcium retained to that ingested. The difference between the calcium found by analysis at the end of the experimental period and that calculated to have been present at 28 days equals the calcium retained.

The calcium utilization factor of the control diet was the greatest, showing the ready availability of the milk calcium, while that of the turnip green diet was about equal to that of the control diet. A much larger number of animals would be necessary to determine whether this small difference was significant. From the calcium utilization factors of the tender-green, collard and kale diets it appears that the calcium of these greens was not utilized quite as well as that of the turnip greens and the milk. The calcium utilization factor of the New Zealand spinach diet was extremely small compared with that of the other diets, equalling only about one-quarter that of the control diet. Apparently, not only was the calcium of the New Zealand spinach unavailable, but also the availability of the calcium of the milk in the diet was reduced.

Although New Zealand spinach (Tetragonia expansa) and spinach (Spinacea oralacea) are not of the same family they resemble one another in respect to their relatively high content of oxalate. Several groups of workers (Fincke and Sherman,

TABLE 3

Calcium content of 60-day-old rats on the various diets

DIET	NUMBER OF RATS	Ca IN BODY AT 60 DAYS		Ca IN BODY AT 28 DAYS (CALCULATED)	Ca RETAINED (CALCULATED)	Ca IN FOOD EATEN	Ca UTILIZATION FACTOR
		Total	Per cent of net weight				
Males							
		gm.	%	*gm.*	*gm.*	*gm.*	
Control	4	1.129 ± 0.134[1]	0.87 ± 0.02[1]	0.361 ± 0.063[1]	0.767 ± 0.079[1]	0.967 ± 0.128[1]	0.80 ± 0.05[1]
Turnip green	6	0.978 ± 0.086	0.91 ± 0.04	0.358 ± 0.045	0.620 ± 0.041	0.808 ± 0.081	0.77 ± 0.03
Tendergreen	6	0.982 ± 0.076	0.90 ± 0.01	0.352 ± 0.047	0.630 ± 0.035	0.850 ± 0.040	0.74 ± 0.03
Collard	6	0.954 ± 0.101	0.93 ± 0.03	0.369 ± 0.036	0.585 ± 0.076	0.846 ± 0.065	0.69 ± 0.05
Kale	3	0.977 ± 0.039	0.91 ± 0.03	0.365 ± 0.038	0.612 ± 0.035	0.866 ± 0.063	0.71 ± 0.01
New Zealand spinach	5	0.410 ± 0.076	0.69 ± 0.05	0.341 ± 0.062	0.069 ± 0.041	0.478 ± 0.029	0.14 ± 0.08
Females							
Control	5	0.985 ± 0.052	0.96 ± 0.03	0.342 ± 0.024	0.679 ± 0.038	0.820 ± 0.060	0.78 ± 0.06
Turnip green	4	0.891 ± 0.051	0.94 ± 0.04	0.319 ± 0.047	0.572 ± 0.018	0.769 ± 0.027	0.75 ± 0.04
Tendergreen	4	0.880 ± 0.070	0.97 ± 0.01	0.333 ± 0.054	0.547 ± 0.046	0.767 ± 0.049	0.71 ± 0.03
Collard	3	0.861 ± 0.016	1.01 ± 0.02	0.320 ± 0.012	0.541 ± 0.006	0.779 ± 0.018	0.70 ± 0.02
Kale	6	0.870 ± 0.058	1.05 ± 0.10	0.345 ± 0.035	0.525 ± 0.027	0.800 ± 0.052	0.66 ± 0.04
New Zealand spinach	3	0.459 ± 0.004	0.84 ± 0.03	0.356 ± 0.054	0.102 ± 0.050	0.486 ± 0.021	0.21 ± 0.10

[1] Average deviation of the mean.

'35; Fairbanks and Mitchell '38; and Tisdall and Drake, '38) have shown that the calcium of spinach is utilized poorly, if at all, by the growing rat and have attributed this to the oxalate content of spinach. Furthermore, Fairbanks and Mitchell ('38) found the calcium of calcium oxalate unutilizabe. About 75% of the calcium in the New Zealand spinach was probably in this insoluble form, for successive extractions of the New Zealand spinach with 2N HAc and 2N HCl dissolved only 25% of its calcium. It is of interest that this is about the same percentage of soluble calcium as that of 30% found by Horwitt, Cowgill and Mendel ('36) to be soluble on in vitro digestion of dried spinach with a pepsin-HCl mixture. Even if all the calcium in the New Zealand spinach diet were in the form of the oxalate, about 50% more oxalate would remain for possible combination with the other calcium in the diet. Mackenzie and McCollum ('37) have shown that potassium oxalate can decrease the utilization of calcium on borderline calcium diets, but not on high calcium diets. Consideration of our results in the light of this work reveals the possibility that the available calcium content of the diet was so lowered with 75% or more of the calcium in the New Zealand spinach bound in the form of unavailable calcium oxalate that the deleterious effect of the remaining oxalate was magnified, resulting in the decreased utilization of the calcium of the milk in the diet.

SUMMARY

In these experiments measurements were made of the utilization of the calcium of various dried greens in diets in which one-half of the calcium was supplied by the green and the other half by milk. These diets were fed to young rats from their twenty-eighth to sixtieth day of age, at which time they were killed and analyzed for calcium. The calcium utilization factor, the ratio of the calcium retained to that ingested, was used as a measure of the utilization of the calcium in the various diets during this period of rapid growth and calcification.

The results showed that the calcium of turnip greens was about as available as that of milk, while that of tendergreen, collards and kale was slightly less available. Not only was the calcium of New Zealand spinach utilized poorly, if at all, but also the utilization of the calcium of the milk in the diet was diminished by the presence of the New Zealand spinach.

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THE GROWTH, ACTIVITY, AND COMPOSITION OF RATS FED DIETS BALANCED AND UNBALANCED WITH RESPECT TO PROTEIN [1]

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INTRODUCTION

A balanced ration is one containing all the necessary nutrients in the proper proportions to promote the maximum bodily performance for which the ration is balanced and one which does not impair any bodily function or injure any internal organ. A ration balanced for growth, therefore, is one which will enable the animal to grow at a maximum rate. While it seems logical to suppose that the better balanced of two rations, for any particular purpose, will be more efficiently utilized as a whole than the less well balanced one, this has not been definitely proved. Also a ration balanced with respect to some particular nutrient may not be the one in which that nutrient is utilized to the greatest possible extent, but the bulk of all nutrients present, possibly best represented by the metabolizable energy content of the ration, probably will be more efficiently utilized than any other combination of nutrients (amounts consumed being the same) which will promote the same retention of the particular nutrient under consideration. For example, in a growing animal it is probable that protein retention will increase as the percentage of protein in the ration increases up to 18 or 20%, but this increase in retention will take place at a decreasing rate of

[1] The experimental data reported here have been taken from a thesis submitted in 1937 to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of doctor of philosophy in animal husbandry. Preliminary reports of this investigation have appeared previously (Hamilton, '35, '37).

efficiency of utilization; however, the efficiency with which the dietary energy is utilized by the animal would probably increase as the ration became better balanced.

The results to be reported here, and in the following paper, were obtained during a study started in July, 1934, concerned with the utilization of the energy of diets balanced and unbalanced with respect to protein for growing rats. The data to be reported in this paper deal with the effects of the percentage of protein in the diet upon the growth, activity, composition of gains, appetite and size of liver and kidneys of young growing male rats. The following paper will be concerned with the effects of the percentage of protein in the diet upon the utilization of its energy, with particular emphasis on the heat increments.

REVIEW OF PREVIOUS EXPERIMENTS

In addition to many observations based on the ad libitum method of feeding, that the growth-promoting values of diets increase as the percentage of protein in the diet increases up to 18 or 20, a few experiments in which a controlled method of feeding was used have demonstrated conclusively that this is so. Hogan and Pilcher ('33), using diets varying in protein content from about 7 to 33%, found that rats on the higher protein diets made better gains than did the rats on the lower protein diets, even though both diets furnished the same amounts of metabolizable energy. Hogan, Johnson and Ashworth ('35) and Johnson, Hogan and Ashworth ('36) compared a 10.0 with a 26.2% protein diet and showed that the rats on the higher protein diet made greater gains and stored more water, protein and energy, but less fat, than did their pair mates on the lower protein diet.

Forbes, Swift, Black and Kahlenberg ('35 a, '35 b) found on feeding rats equicaloric amounts of diets containing, respectively, 10, 15, 20 and 25% of protein, that with increased protein, up to at least 20%, there was an increase in total gains, an increase in protein stored and a decrease in fat gained per unit of protein gained. In a preliminary report, Mitchell and

Hamilton ('36) have also shown, with rats and with pigs, that the better balanced a ration is with respect to protein (so long as the percentage does not exceed the minimum required for maximum rate of growth), the greater is its growth-promoting value, and the gains of the higher protein-fed animal contained more protein and less fat per unit of protein gained than did those of comparable animals fed equicaloric amounts of a lower protein ration.

Forbes, Voris, Bratzler and Wainio ('38) fed quadruplets of rats diets containing 25, 30, 35 and 45% protein and found that equicaloric amounts of these diets produced decreasing rates of gains as the percentage of protein increased above 25. Mitchell and Smuts ('32) have also shown by the paired-feeding technic that the minimum percentage of meat protein capable of supporting maximum growth rate in rats was between 18 and 20%.

Concerning the effect of the percentage of protein in the diet upon the voluntary activity of rats, Slonaker ('31) concluded from ad libitum feeding tests that diets containing between 14 and 18% protein were most conducive to maximal spontaneous running. Forbes, Swift, Black and Kahlenberg ('35 a) concluded that the greater growth-promoting abilities of diets better balanced with respect to protein was due to a decreased activity of the rats fed the better balanced diets. This conclusion was made without any attempt to measure the activities of the rats, however.

As to the effects of the percentage of protein in the diet upon its palatability or upon the appetite of the animal, it is assumed all too frequently that the appetites of the experimental animals are infallible indices of the nutritive completeness of the diets fed. Fortunately for the very large number of ad libitum feeding experiments, this assumption seems to be generally true, but unfortunately this is not always the case. Beadles, Braman and Mitchell ('30) and Mitchell and Beadles ('30) demonstrated that the growth-promoting value of both cooked and uncooked garden peas could be improved by the addition of small amounts of cystine. How-

ever, in the cooked pea experiment there were fifty-nine food refusals by the rats on the deficient diet and eighty by the rats receiving the same amount of the supplemented diet. In the raw pea test, there were but twenty-one food refusals by rats receiving the deficient diet and forty-seven by the rats on the supplemented diet. Mitchell and Smuts ('32) also reported two cases, one with a meat protein diet and another with a corn protein diet, in which the rats receiving the diets with the lower growth-promoting value apparently had the better appetites.

PLAN OF INVESTIGATION

The general plan of this investigation consisted of a series of experiments on white rats involving paired-feeding, carcass analyses and metabolism studies. The experimental diets were complete and alike in all respects except for the percentage of protein which was varied from 4 to 54. The protein was supplied in the form of dried whole egg. Increases in the percentages of protein were accomplished at the expense of corn starch. The ingredients of the diets were as follows:

Constant factors	
Salts[2]	5.00%
Agar agar	1.00%
Cod liver oil[3]	3.00%
Filtered butterfat	8.00%
Variable factors	
Sucrose	10.00%
(This percentage was constant in all diets except the 54% protein diet, in which it was 2.35%)	
Purified corn oil[4]	8.00%
(Varied slightly so as to make a total of 19.4% fat in all diets)	
Cornstarch[5]	59.13% to 0%
Extracted whole dried egg[6]	5.87% to 69.80%

[2] Wesson ('32) slightly modified so as to contain traces of Co, Zn and Mn.
[3] E. L. Patch Co., Boston, Mass.
[4] Mazola.
[5] Contains no more than traces of nitrogen. A. E. Staley Mfg. Co., Decatur, Ill.
[6] Phenix-Kraft Cheese Co., Chicago, Ill. Extracted with ether until less than 1% fat remained.

The B vitamins were supplied in the form of Harris yeast concentrate, each rat receiving 0.5 mg. daily per gram of body weight. This amount of yeast concentrate was selected after preliminary paired-feeding experiments, using both high and low protein diets containing 20% fat, indicated that the above stated amount was ample for maximum growth rate.

The synthetic diets studied contained the following approximate percentages of protein (T.N. $\times$ 6.25): 4, 8, 12, 16, 18, 20, 22, 30, 42 and 54. All tests were of a comparative nature; the following comparisons having been made: 4 versus 8 (two tests), 8 versus 12 (two tests), 12 versus 16 (two tests), 8 versus 18, 16 versus 20, 18 versus 22, 22 versus 42, 20 versus 54 (two tests), and 20 versus 54 (cooked egg). The diets were compared in the following respect: 1) ability to produce gains in weight and in body length; 2) ability to produce retentions of energy and of nitrogen; 3) effects upon the appetite; 4) effects upon the voluntary activity of rats; 5) their contents of digestible nitrogen and metabolizable energy, and 6) in a few cases, their effects upon the weights of livers and kidneys in rats.

The comparative growth-promoting abilities of the various diets were determined by a series of fourteen 5- to 24-week paired-feeding tests. In each test, eight pairs of brother rats were used. Inbred white rats, approximately 4 weeks of age and weighing about 50 gm. at the start of the test, were paired as to litter, age, sex and weight. With few exceptions, notably the tests in which low-protein diets were fed, all tests continued until the rats had gained between 150 and 175 gm. At the termination of each test, all rats were etherized, their nose-to-anus lengths and empty body weights determined. Eventually all carcasses were analyzed for their total nitrogen and gross energy contents. While body weight and length increases were used as indications of the comparative growth-promoting abilities of the diet, the amounts of protein stored were used as the final criterion.

The rats on paired-feeding tests were kept in small four-compartment wire cages each compartment measuring 2.5 $\times$

5 (or 7) × 7 inches. These small cages were used in order to restrict the activity of the rats as much as seemed practicable and it was hoped also that the activity of pair-mates would be more nearly equal. All rats were kept in an air-conditioned room, the temperature of which was maintained at 28 ± 1°C. and the humidity at 43 ± 5%. The only work not done in the air-conditioned room was that in which the revolving cages were used in order to compare the voluntary activity of paired rats receiving the different diets.

In three paired-feeding tests, the usual paired-feeding technic was followed, i.e., pair-mates were fed the same amounts of food daily. In all other tests, a modified procedure was employed. This modified procedure consisted in the daily feeding of such amounts of the diets being compared that equal daily gains in weight resulted. This latter procedure, while furnishing quantitative data relative to the growth-promoting abilities of the diets used, had the added advantage that the maintenance requirements of pair-mates would be very nearly the same throughout the test. The digestibility of the protein and the metabolizable energy content of each diet were determined by the usual method. In most cases these values were determined after the rats had been on the paired-feeding test for at least 3 weeks. During the 14-day collection period, the rats were kept in glass-bottomed metabolism cages to enable accurate urine collections. Gross energy determinations were made on the urines and feces after drying at low temperatures.

The voluntary or running activity of three series of rats was determined by keeping, for several weeks, each series of eight pairs of rats in a battery of sixteen identical revolving cages (compare Mitchell, '32–'33) each of which was equipped with a revolution counter.

In the following presentation and discussion of results, each datum represents the average of at least eight values obtained on as many individual rats, unless otherwise made clear to the contrary. All data were analyzed statistically.

Conclusions made are based upon the probability of the existence or non-existence of differences as found by the application of Student's ('08) method of analyzing paired data. A difference between two averages was judged to be significant when the value of P was 0.03 or less.

RESULTS

Digestibility of the nitrogen and of the energy, and the metabolizable energy of diets containing different percentages of protein. Table 1 gives the average nitrogen and energy contents of the diets used. In table 2 are summarized the data obtained from the balance experiments. These data seem to

TABLE 1

Average composition of diets

Approximate protein content	*Total nitrogen*	*Crude protein (T.N. × 6.25)*	*Gross energy*
%	%	%	*Calories per gram*
4	0.668	4.17	4.641
8	1.315	8.22	4.722
12	1.908	11.93	4.752
16	2.553	15.96	4.846
18	2.851	17.82	4.800
20	3.259	20.37	4.848
22	3.549	22.18	4.886
30	4.688	29.30	5.083
42	6.647	41.54	5.238
54	8.684	54.28	5.480

justify the following conclusions: increasing the percentage of whole egg protein from 4 to 54, by direct substitution for starch in the diet: 1) increased the gross energy; 2) increased the percentages of gross energy and of nitrogen ingested that appeared in the urine; 3) increased the metabolizable energy but slowly; 4) had no effect upon the percentage of gross energy which was metabolizable until 30% or more protein was present, when a gradual decrease occurred; 5) caused a gradual decrease in the ratio of energy to nitrogen in the urine, and 6) had no consistent effect upon the digesti-

TABLE 2

Energy and nitrogen utilized and wasted from the various diets

Approximate protein content, %	*4*	*8*	*12*	*16*	*20*	*30*	*42*	*54*
Energy								
Gross, Calories per gram of diet	4.641	4.722	4.752	4.846	4.848	5.083	5.238	5.480
Metabolizable,[1] Calories per gram of diet	4.412	4.382	4.429	4.606	4.624	4.702	4.754	4.842
S[2]	0.094	0.053	0.022	0.007	0.023	0.028	0.017	0.051
Metabolizable,[3] Calories per gram of diet	4.376	4.345	4.363	4.505	4.532	4.560	4.598	4.711
S	0.106	0.043	0.024	0.013	0.019	0.019	0.016	0.090
Metabolizable,[1] percentage of gross	95.2	94.4	93.6	94.4	93.4	91.6	90.5	87.9
S	0.50	0.40	0.16	0.21	0.49	0.55	0.30	0.80
Digestible, percentage of gross	96.4	95.8	95.3	96.3	96.0	95.3	95.1	94.3
S	0.4	0.4	0.2	0.1	0.2	0.2	0.2	0.4
Fecal, percentage of gross	3.62	4.16	4.67	3.70	3.97	4.66	4.99	5.66
S	0.44	0.37	0.18	0.17	0.23	0.17	0.16	0.41
Urinary, percentage of gross	1.14	1.43	1.68	1.93	2.62	3.71	4.50	6.41
S	0.27	0.37	0.15	0.06	0.21	0.51	0.25	0.77
Nitrogen								
Digested, apparent, %	75.9	81.4	80.0	90.5	88.5	90.9	92.5	88.1
S	3.9	1.7	2.8	0.5	2.2	0.6	0.2	4.0
Digested, true,[4] %	100[4]	93.5	88.7	96.9	93.7	94.5	94.9	90.0
Fecal, percentage of N intake	24.1	18.5	19.9	9.54	11.48	9.06	7.54	11.93
S	4.0	1.71	2.81	0.52	2.18	0.64	0.24	4.00
Urinary, percentage of N intake	57.3	46.1	35.0	38.4	50.8	68.4	61.4	67.9
S	13.5	13.1	2.80	4.38	8.3	3.5	3.0	7.1
Energy per gram N in urine, Calories	12.88	10.56	11.53	9.39	7.93	5.90	5.64	6.01
S	2.73	2.11	0.60	1.03	0.79	0.83	0.70	0.80
Number of determination	16	12	4	8	8	8	8	8

[1] Uncorrected for nitrogen balance.

[2] $S = \sqrt{\frac{(A - X)^2}{n - 1}}$

[3] Corrected for nitrogen balance.

[4] Assuming the protein of the 4% diet to be completely digested. The average fecal N per gram of 4% diet was 1.92 mg. and this was assumed to be the metabolic N per gram on all other diets.

bility of either food energy or food nitrogen. It may be calculated that, increasing the protein in the diet from 4 to 54%, the gross energy increased 15.3% while the metabolizable energy increased but 8.8%. The per cent of the gross energy which was metabolizable decreased less than 8%, the greatest decrease occurring between the 30 and 54% protein diets.

The effect of the percentage of protein in the diet upon its growth-promoting value. The data relative to the growth-promoting value of the various diets are summarized in table 3. Using nitrogen retention as a measure of the growth-promoting abilities of the diets, the data show that, as the percentage of protein increases from 4 to some percentage between 12 and 16, the growth-promoting values of the diets also increase. Increasing the percentage of protein in the diet from 16 to 54 caused no further increase in protein retention by rats increasing in weight from 50 to about 200 gm.

The final body lengths of paired rats, gaining in weight at the same rate, increased with the percentage of protein in the diet until 12% was present, remained equal for diets containing between 12 and 42% protein and then decreased when 54% protein was present.

Using the amounts of metabolizable energy required to produce equal rates of gain in paired rats as a measure, the growth-promoting abilities of the diets increased with the percentage of protein from 4 to between 12 and 16, remained constant between 16 and 22 and then decreased when 42% or more protein was present. Using the ratio of total metabolizable energy intake of the low-protein-fed rats divided by that of the high-protein-fed rats making the same daily gains, the efficiencies of the various diets may be expressed as follows:

4 versus 8	1.220 : 1	16 versus 20	1.003 : 1
8 versus 12	1.155 : 1	18 versus 22	1.006 : 1
8 versus 18	1.207 : 1	22 versus 42	0.946 : 1
12 versus 16	1.029 : 1	20 versus 54	0.916 : 1

The minimum percentage of whole egg protein, capable of producing maximum growth, seems to lie between 12 and 16.

TABLE 3

The growth-promoting ability of diets containing different percentages of protein (averages for at least eight pairs of rats)

	4 versus 8[1]		8 versus 12		8 versus 18		12 versus 16		16 versus 20		18 versus 22		22 versus 42		20 versus 54	
Total food consumed—																
Gm.	511	417	886	776	598	480	641	607	475	469	421	413	466	469	411	415
P[2]	<0.0001		<0.0001		<0.0001		0.0001		0.19		0.10		0.32		0.23	
Total gains—																
Gm.	42.5	43.5	188.0	189.9	154.4	152.4	180.0	180.8	163.3	161.6	154.0	153.6	153.0	152.0	140.8	136.1
Final body length—																
mm.	162.9	165.5	215.8	217.6	208.8	209.1	216.0	217.3	209.6	212.0	208.0	208.5	209.1	210.9	208.4	205.9
P	0.0002		0.056		0.037		0.19		0.073		0.24		0.085		0.030	
Total metabolizable energy intake[3]—																
Cals.	2225	1824	3931	3402	2675	2217	2820	2740	2148	2142	1909	1898	2133	2249	1920	2095
P	<0.0001		<0.0001		<0.0001		0.0088		0.42		0.37		0.0041		0.0092	
Total nitrogen gained—																
Gm.	1.44	1.70	5.76	6.19	4.56	4.92	5.34	5.51	5.12	5.17	4.76	4.69	4.73	4.62	4.86	4.72
P	<0.0001		0.0004		0.0013		0.038		0.33		0.30		0.16		0.14	
Energy gained per gram N gained—																
Cals.	87.2	62.2	101.0	88.4	117.1	97.8	110.5	102.0	104.0	98.7	99.1	100.5	109.4	105.1	66.6	61.6
P	<0.0001		0.0012		0.0046		0.0048		0.18		0.4		0.29		0.0036	

[1] Approximate percentage of protein in the diets compared.

[2] Probability that the difference between the averages was due to chance, according to 'Student,' ('08). A P-value of 0.03 or less is considered highly significant.

[3] Corrected to exactly equal gains.

While the data for the entire feeding period of the 16 versus 20% protein diets indicated that the 20% diet was no better than the 16, the daily food records indicated very strongly that, until the rats weighed about 125 gm., the 20% protein diet had a higher growth-promoting value than the 16. For very young rapidly growing rats, the minimum percentage of a high quality protein capable of supporting a maximum growth rate is probably near 20%. As to the maximum percentage of egg protein capable of producing maximum growth, 54% seems to be excessive. Although in the 22 versus 42% protein comparison pair-mates made the same gains in total weight, body length, nitrogen and energy on the same amounts of food, more metabolizable energy was required by the 42% protein-fed rats than was required by their 22% protein-fed pair mates. Also, as will be shown in the following paper, the heat increment of the 42% protein diet is greater than that of a 20% protein diet, while the heat increment of a 30% protein diet was no greater than that of a 20% protein diet. It seems, therefore, that the upper limit of whole egg protein capable of supporting a maximum rate of growth in rats is between 30 and 42%.

In general, the effects of the percentage of protein in the diet upon its growth-promoting ability, as reported here, are in agreement with the findings of other workers. It should be emphasized that the range of protein percentages over which a diet will be balanced will depend upon the nature of the protein and the requirements of the animal.

The effect of the percentage of protein in the diet upon the composition of the gains. In table 4 are summarized the data relative to the effects of the percentage of protein in the diets upon the composition of gains in rats. The compositions of the gains were calculated from the chemical analyses of the rats at the end of the feeding tests and from the composition of control rats slaughtered at the time the feeding test was started.

Data were obtained on eleven series of rats. In each series, eight or more pairs of rats were used. In three series, the

TABLE 4

The effect of the percentage of protein in the diet upon the composition of gains in rats. (Figures represent data from at least eight pairs of rats)

Diets compared	Total gains		Total nitrogen gained			Total energy gained				Energy gained per gram T.N. gained			
	gm.	gm.	M	s	P	Calories	M	s	P	Calories	M	s	P
4 (= food)	33.1	0.850				140.9				165.7	86.5	30.6	0.0001
8	67.0	2.314	1.464	0.413	<0.0001	183.3	42.4	36.3	0.0088	79.2			
8 (= food)	67.2	2.577				192.8				74.8	10.6	2.54	<0.0001
12	79.3	3.154	0.577	0.312	0.0009	202.6	9.8	17.4	0.092	64.2			
12 (= food)	85.3	2.991				288.7				96.5	4.0	11.2	0.19
16	88.4	3.244	0.253	0.122	0.0005	300.2	11.5	30.6	0.184	92.5			
4 (= gain)	42.5	1.443				125.8	20.1	18.7	0.013	87.2	25.0	6.57	<0.0001
8	43.5	1.699	0.256	0.069	<0.0001	105.7				62.2			
8 (= gain)	188.0	5.757				581.4	34.3	43.8	0.039	101.0	12.6	7.17	0.0012
12	189.9	6.187	0.430	0.202	0.0004	547.0				88.4			
8 (= gain)	154.4	4.564				543.2	52.6	47.9	0.011	117.1	19.3	14.3	0.0046
18	152.4	4.922	0.358	0.207	0.0013	481.6				97.8			
12 (= gain)	180.0	5.345				590.4	28.2	27.1	0.014	110.5	8.5	6.33	0.0048
16	180.8	5.511	0.166	0.211	0.038	562.2				102.0			
16 (= gain)	163.3	5.120				532.6	27.8	44.1	0.071	104.0	5.3	13.2	0.18
20	161.6	5.166	0.046	0.271	0.33	504.8				98.7			
18 (= gain)	154.0	4.756	0.062	0.297	0.30	471.2				99.1			
22	153.6	4.694				471.7	0.5	39.0	0.42	100.5	1.4	13.4	0.40
22 (= gain)	153.0	4.734	0.114	0.254	0.16	517.7	32.3	50.2	0.084	109.4	4.3	16.4	0.29
42	152.0	4.620				485.4				105.1			
20 (= gain)	140.8	4.856	0.135	0.246	0.14	277.7	26.7	21.8	0.0025	66.6	5.0	4.29	0.0036
54	136.1	4.721				251.0				61.6			

diets used varied in their percentage of protein from 4 to 16, and pair-mates were fed equal amounts of feed daily. In eight series, in which the protein contents of the diets varied from 4 to 54%, the pair-mates were fed such amounts of feed that equal daily gains were obtained. Data of similar significance were obtained from both types of feeding, although it seems reasonable to suppose that the data obtained from the equal gain procedure have the more exact significance in this connection because the pair-mates at all times throughout the test were of the same weight as well as of the same sex, age and parenthood.

From the data presented in table 4, the following conclusions seem justified: from 4 to 16% dietary protein the gains of rats on the higher plane of protein intake contained more nitrogen, while the gains of rats, increasing in weight at the same rate on a lower plane of protein intake, contained more fat. Diets containing from 16 to 42% protein produced gains of similar composition. In the 20 versus 54% protein comparison, while there were no significant differences in the amounts of nitrogen gained by pair-mates, the 20% protein-fed rats put on gains which were fatter.

The effect of the percentage of protein in the diet upon the appetite and the activity of the animal. In all paired-feeding tests records of daily food refusals were kept and in this manner evidence as to the relative effects of the diets upon the appetites of the rats was obtained. In three comparisons by the equal-daily-food technic and in four comparisons by the equal-daily-gain procedure, the amounts of food eaten by pair-mates daily were either exactly equal or approximately equal. The data in table 5 indicate a direct correlation between the growth-promoting values of the diets and their appetite stimulating effects. In all comparisons with diets containing less than 16% protein, the diet having the higher protein content also had the greater appetite stimulating effect. Diets containing between 16 and 22% protein were not only of equal growth-promoting values, but were also equal with respect to their effects upon the appetites of the rats.

TABLE 5

Effect of the percentage of protein in the diet upon the appetite of the animal

	4 versus 8[1]		*8 versus 12*[1]		*12 versus 16*[1]		*16 versus 20*[2]		*18 versus 22*[2]		*22 versus 42*[2]		*20 versus 54*[2]	
	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High	Low	High
Total food consumed														
Gm.	731	731	335	335	273	274	475	469	421	413	466	469	411	415
P[3]	<0.0001		<0.0001		<0.0001		0.19		0.10		0.32		0.23	
Diet having the higher growth-promoting value	8		12		16		equal		equal		22		20	
Average number of food refusals	81.1	4.0	22.8	7.8	12.1	4.3	2.4	5.4	10.5	14.2	6.9	28.9	1.5	33.6
P[3]	<0.0001		0.029		0.024		0.16		0.16		0.0038		0.0002	

NOTE: The average figures in this table represent eight animals.

[1] Equal-daily-food procedure.

[2] Equal-daily-gain procedure.

[3] Probability that the difference was significant, according to 'Student' ('08).

In the 22 versus 42 and the 20 versus 54% protein comparisons, the higher protein diet in each case markedly depressed the appetites of the rats, and also in each case the higher protein diet was of less value for growth purposes. While these results are in agreement with the frequent assumption that the appetite of an experimental animal is an infallible index to the nutritive completeness of a diet, it should be pointed out that this is not always the case.

TABLE 6

The effect of the percentage of protein in the diet upon the activity of the animals consuming it. (Figures represent thousands of revolutions)

Pair Number	4 versus 8[1]		8 versus 12[1]		20 versus 54[2]	
	Low	High	Low	High	Low	High
1	230.0	413.4	431.1	371.7	234.5	105.0
2	447.6	487.8	347.3	379.7	131.0	105.5
3	504.2	455.6	235.1	353.3	208.9	251.4
4	429.4	198.2	391.9	137.6	268.4	83.6
5	319.4	262.6	45.8	418.0	239.4	166.5
6	267.4	378.5	272.0	232.0	266.7	117.5
7	323.2	437.3	226.8	459.7	170.2	173.2
8	455.5	433.8	389.6	327.9	272.6	247.6
Average	372.1	382.0	292.4	335.0	224.0	156.2
M		9.9		42.6	67.8	
S		122		183	74.9	
P		0.4		0.282	0.023	

[1] Equal food daily.
[2] Equal gain daily (also approximately equal food).

In order to determine whether or not the percentage of protein in the diet affected the voluntary activity of the rats, three series of paired rats were kept for several weeks in a battery of sixteen identical revolving cages, each cage being equipped with a revolution counter. The three series in which the voluntary or running activity was measured were the 4 versus 8 (= food), 8 versus 12 (= food) and the 20 versus 54 (= gain) % protein diets. The data are given in table 6. According to these data, percentages of protein varying from 4 to 12 had no differential effect upon the running activity of the rats. It therefore seems unlikely that diets better balanced

with respect to protein would do so. Rats receiving a 54% protein diet, however, were less active than rats growing at the same rates on approximately the same amounts of a 20% protein diet.

Forbes, Swift, Black and Kahlenberg ('35 b), working also on the problem of food energy utilization as affected by the percentage of protein, attempted to explain their results on the assumption of a decreased voluntary activity of the rats as the diets became better balanced with respect to protein. The data reported here do not support this assumption.

TABLE 7

The effect of the percentage of protein in the diet upon the weights of the livers and kidneys of the rats consuming it

	LIVER WEIGHTS		KIDNEY WEIGHTS					
	20 versus 54 (= gain)		8 versus 18 (= gain)		22 versus 42 (= gain)		20 versus 54 (= gain)	
	Low	High	Low	High	Low	High	Low	High
	gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.
Average	7.25	8.04	1.47	1.63	1.65	1.87	1.59	1.83
M		0.79		0.16		0.22		0.24
S		0.60		0.128		0.12		0.168
n		11		8		8		11
P		0.0006		0.0066		0.0009		0.0005

The effect of the percentage of protein in the diet upon the liver and kidney weights of animals consuming it. That high protein diets produce hypertrophy of the kidneys of the animals consuming them is well established, although the effects upon the liver are less certain. In most previous work of this kind, comparisons were made with so-called normals. In this study, having at hand a number of rats of the same age, sex, size, parenthood and having grown at the same rate on diets which differed only in their contents of protein, it seemed that conditions were ideally controlled to study the effects of the percentage of protein in the diet upon liver and kidney weights. The data obtained upon this point are summarized in table 7, and from these data it is concluded that there is a direct relationship between the percentage of protein in the diet of growing rats and the weights of their livers and kidneys.

CONCLUSIONS

1. Increasing the percentage of whole egg protein from 4 to 54 by the direct substitution of starch in the diet of rats 1) increased the metabolizable energy but slowly; 2) had no significant effect upon the percentage of gross energy which was metabolizable until 30% or more protein was present when a gradual decrease occurred; 3) caused a gradual decrease in the ratio of energy to nitrogen in the urine, and 4) had no consistent effect upon the digestibility of either food energy or food nitrogen.

2. Increasing the percentage of protein from 4 to 16% in the diet of growing animals, increased the growth-promoting value of the diet. Diets containing between 16 and 30% of a good quality protein were of equal growth-promoting value, and as the protein increased above 30%, the growth-promoting value decreased. For male rats weighing less than 125 gm., the minimum percentage of protein in a well-balanced diet may be as high as 20.

3. Total gains in weight are not as good a criterion of growth as is gain in protein and gain in body length.

4. The appetites of rats consuming diets containing less than 16 or more than 22% protein were adversely affected.

5. The voluntary activity of rats was unaffected by the protein content of the diet until an excess was present when the activity was decreased.

6. The composition of the gains of growing animals was readily affected by the percentage of protein in the diet. Diets containing less than 16% protein produced gains having more fat but less protein than did well-balanced diets having a higher percentage of protein. Diets containing between 16 and 42% protein produced gains of approximately the same composition. A high protein (54% whole egg protein) diet produced gains with less fat than did a 20% protein diet.

7. The weights of the kidneys and the livers of growing rats are influenced by the amounts of protein consumed, the relationship being a direct one.

The writer is indebted to Prof. H. H. Mitchell for many valuable suggestions made throughout the progress of this investigation.

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THE HEAT INCREMENTS OF DIETS BALANCED AND UNBALANCED WITH RESPECT TO PROTEIN [1]

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INTRODUCTION

That the nutritive balance of a diet was a determining factor in the efficiency with which an animal utilizes the dietary energy, was first recognized, so far as the writer is aware, by Møllgaard ('23). He stated, "The quotient of production (the proportion of the net energy of the ration contained in the protein component) of any feeding-stuff is substantially determining the extent to which the net energy of the feeding-stuff may be utilized in production of milk."

A clear and definite statement of this relationship was made by Mitchell ('24) who, in commenting upon some experiments of Osborne and Mendel, stated (loc.cit., footnote, p. 447) "These experiments suggest an interesting relation, hitherto uninvestigated as far as the author is aware, between the balance of nutrients in a ration and the utilization of its energy." The same idea was again expressed by Mitchell and Carman ('26) who obtained as much as 40 to 50% greater gains on the same amount of food in both chicks and rats by substituting 1% sodium chloride for 1% corn in a salt-deficient ration. They stated (loc.cit., p. 177): "The growth data of

[1] The experimental data reported here have been taken from a thesis submitted in 1937 to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of doctor of philosophy in animal husbandry. Preliminary reports of this investigation have appeared previously (Hamilton, '35, '37).

this experiment afford a striking demonstration of the fact that the utilization of food energy by growing animals may be greatly impaired by an improper balance among indispensable dietary factors. The addition of 1% NaCl to ration A has evidently greatly improved the utilization of its available energy, probably by decreasing its stimulating effect on heat production, i.e., its specific dynamic effect, and possibly to some extent by decreasing the muscular activity or restlessness of the rat itself."

In 1928 appeared the first of a series of important papers dealing with and apparently developing independently a similar conception by Forbes and associates. The authors of this first paper were Forbes, Braman and Kriss ('28).

In the second paper of this series, Forbes, Braman and Kriss ('30) made the following statement (loc.cit., p. 65): "In the light of these facts it may be there are, in reality, different net-energy values of feedingstuffs—for growing animals at least—in accord with different nutritive ratios in which the feedingstuffs are combined." And confirmation of this idea was obtained by Forbes, Braman, Kriss and Swift ('31) when they found that the heat increment of corn fed alone at a maintenance level was much greater than that indirectly computed for corn fed at the same level in combination with alfalfa hay. Similar evidence was presented by Forbes, Braman, Kriss and Swift ('33). Other papers concerned with the development of this idea by the Pennsylvania workers have appeared under the authorship of Forbes ('29, '33 a, '33 b); Forbes and Kriss ('32 a, '32 b, '32 c); and by Forbes, Kriss and Miller ('34).

Other workers also have apparently and independently arrived at the conclusion that the utilization of dietary energy is increased by improving the balance of the nutrients in the diet. Thus Fraps ('31, p. 26) states: "The addition of a proteid feed to an unbalanced ration increases the utilization of the energy of the entire ration. The productive energy of a feed in an unbalanced ration is lower than it is in a balanced ration," and "A proteid feed added to an unbalanced ration

has an effect greater than its own productive energy, since it increases the utilization of the other feeds to which it is added. In 1929, Møllgaard ('29, p. 165) presented evidence in confirmation of his 1923 conception when he found that the utilization of dietary energy for milk production was greatest when the ratio of protein energy to carbohydrate and fat energy in the ration approximated the corresponding ratio in milk, i.e., between 0.14 and 0.23. Hansson ('29, p. 53) calculated the relative nutritive values of protein for fattening and for milk production and found that 1 kg. protein for fattening had a starch value of 0.94, while for milk production 1 kg. protein had a starch value of 1.43 kg.

Further evidence is afforded by numerous controlled-feeding experiments in which greater utilization of the energy of diets better balanced with respect to salt, phosphorus, vitamin B, vitamin A, vitamin D, vitamin G, cystine, lysine and protein has been observed. (References to these investigations may be found in a paper by Hamilton, '35.)

Observations of uncommonly low or decreased 'specific dynamic effect' of nutrients or diets, when ingested by animals whose requirements for these nutrients or diets are intensified, are of similar significance. Numerous such observations may be found in the literature (compare Hamilton, '35) and while many lacked proper control, the mass of such observations constitutes strong evidence that the specific dynamic effects of separately ingested nutrients or the heat increments of diets are not constant characteristic values but are variable —decreasing as the metabolic mixture more nearly satisfies the animal's requirements.

Richardson and Mason ('23) obtained some interesting results in this connection. Working with diabetics, they found that a mixed diet, containing the same quantities of protein, fat and carbohydrates calculated to have been oxidized under basal conditions, if divided into portions and administered every 2 hours, produced no increase in the heat production over the basal level. Concerning the significance of these results, Lusk ('31) in one of his last papers said, "We may con-

clude from this evidence that, if a mixed diet be so constituted as to conform to the exact needs of the tissues and be slowly introduced by absorption from the intestines, its specific dynamic action is virtually negligible . . . ."

Recent studies concerned with the effect of the amount of food consumed upon the utilization of its energy have demonstrated that the heat increment per unit of food is not constant but dependent, at least over certain ranges, upon the amount of food consumed. Experiments of this nature have been reported by Gigon ('11) in a human subject, by Forbes, Braman and Kriss ('28, '30) and by Mitchell and Hamilton ('32) in steers, by Wiegner and Ghoneim ('30) in a rabbit and by Forbes, Kriss and Miller ('34) in rats.

The effect of the percentage of protein in the diet upon the utilization of its dietary energy, has been reported by Johnson, Hogan and Ashworth ('36), Forbes, Swift, Black and Kahlenberg ('35) and Forbes, Voris, Bratzler and Wainio ('38) in rats; by Mitchell and Hamilton ('36) in pigs, and by Hammond, Hendricks and Titus ('38) in chickens.

The purpose of the experiments to be reported here was to test the validity of a conception, definitely stated by Mitchell ('34), that the better balanced a ration is in satisfying an animal's requirements, the smaller will be its heat increment and the greater will be its net energy value. Specifically, this series of experiments was concerned with the heat increments and the net energy contents of diets containing varying percentages of protein when fed to satisfy the requirements of growth in young albino male rats.

PLAN OF INVESTIGATION

The general plan of this investigation, which was started in July, 1934, consisted of a series of experiments on white rats, involving paired-feeding, carcass analyses, metabolism tests and determinations of the respiratory exchange during both the absorptive and post-absorptive states. The experimental diets were complete and alike in all respects, except for the percentage of protein which was varied from 4 to 54.

The protein was supplied in the form of dried, extracted whole egg and increases in the percentages of protein were accomplished at the expense of cornstarch. While this procedure had the effect of gradually increasing the gross energy of the diets as the percentage of protein was increased, the metabolizable energy increased but slowly (compare preceding paper).

All tests were of a comparative nature, and the various diets were compared in a number of respects. In the preceding paper, the effects of the percentage of protein in the diet upon the growth-promoting value, the digestibility of the nitrogen and energy and the metabolizable energy contents of the diets, the appetites, activity and composition of the gains in rats were reported. The data to be reported here are concerned with the effects of the percentage of protein in the diet upon its heat increment and net energy value when fed to growing rats. The diets studied contained the following approximate percentages of protein (T.N. $\times$ 6.25): 4, 8, 12, 18, 20, 30, 42 and 54. Respiratory exchange of rats during both the absorptive and post-absorptive states was made on all rats in the following comparisons: 4 versus 8, 4 versus 12, 8 versus 18, 20 versus 30, 20 versus 42 and 20 versus 54% protein diets. Eight pairs of rats of the same age, sex and parenthood were used in most comparisons. Details of diets, methods of feeding, housing, etc. are described in the preceding paper.

The rats were housed in small four-compartment wire cages in order to restrict their activity, and it was hoped also that the activity of pair-mates would be more nearly equal. All rats were kept in an air-conditioned room, the temperature of which was maintained at 28 $\pm$ 1°C. and the humidity at 43 $\pm$ 5%. All respiration studies were also carried out in this same room. The method of feeding, which was adopted in all series reported here, was a modified paired-feeding technic in which pair-mates were fed, not necessarily the same amounts of food daily, but such amounts that equal daily gains resulted. This procedure has the advantage that since

pair-mates were always of the same weight, their maintenance requirements would be approximately the same, and, in addition, heat productions could be compared directly; or if comparisons were made on the basis of body surface, the uncertainties of any surface area formula would be negligible.

The heat increment, as used in this study, was the heat production above the basal for 17 to 19 hours immediately following the ingestion of 5 gm. (4 in a very few cases) of diet. The basal heat production of each rat was determined in a 3- to 5-hour test which followed the heat increment test. The basal tests were therefore begun after the rat had fasted at least 19 hours. All basal tests were run between the hours of 11 A.M. and 5 P.M. All rats were fasted 24 hours previous to the test meal offered in the heat increment test, and the rat was allowed a maximum of 15 minutes in which to consume entirely the test meal.

The Haldane (1892) gravimetric procedure was used for the heat increment tests and the Benedict ('30) multiple-unit respiration apparatus, modified so as to measure the CO_2 production gravimetrically, was employed in the basal tests. In both types of apparatus, a kymographic record of activity was obtained, and all experiments in which evidence of more than minor movements of the rat were obtained were repeated.

The heat increments of diets containing 4, 8, 18, 20 and 54% protein were determined, using rats that had been on a paired-feeding test for at least 3 weeks. Only three pairs of rats in the 4 versus 8% protein series would consume sufficient food in the allotted 15 minutes to make a satisfactory test, although eight pairs were used in all other tests. The heat increments of diets containing 4, 12, 20, 30 and 42% protein were also determined using male rats weighing between 125 and 150 gm. and maintained on a good stock diet for 7 to 14 days. The comparisons made in this latter series were the 4 versus 12, 20 versus 30 and 20 versus 42% protein diets. In making these comparisons, the heat increments of both diets being compared were made on each of eight rats, and

on alternate rats the order in which the diets were tested was reversed.

RESULTS

The effect of the percentage of protein in the diet upon its heat increment. In table 1 are summarized the data concerned with the influence of the percentage of protein in the diet upon its heat increment. In all except two cases the data

TABLE 1

The effect of the percentage of protein in the diet upon the heat increment

Protein in diets compared %	*Average heat increment* Small calories *per gram*	*M*	*S*	*N*	*P*
4	415	111	33	3	0.021
8	304				
4	330	95	95	8	0.017
12	235				
8	500	365	205	8	0.0010
18	135				
20	94				
30	158	64	116	8	0.12
20	232				
42	492	260	191	8	0.0044
20	152				
54	625	473	361	8	0.0047
54 (Cooked)	784			4	

Note: The heat increment is the total increase, above the basal, in the heat production of rats for a 17- to 19-hour period immediately following the consumption of 5 gm. of the experimental diet. (To a few pair-mates 4 gm. of diet were offered.)

represent averages from eight pairs of rats. Average differences and probabilities of their significance, according to 'Student's' ('08) method, are also given.

Reference to the table will show that, in all three comparisons made within the range of 4 to 18% protein, the diet containing the lower percentage of protein had the higher heat increment. Diets containing 20 and diets containing 30% protein produced about the same heat increment, and the

values for these two diets were very low and of about the same magnitude as the 18% protein diet. Diets containing 42% or more protein caused increasingly greater heat increments when fed to growing rats.

As the standard deviations indicate, rather wide differences in the heat increments between pair-mates in the same series were sometimes obtained. This is to be expected since very

TABLE 2

Heat increments of the 4 and 12% protein diets, each fed to the same rat, and the heat increments of the 20 and 42% protein diets fed to paired rats (results expressed in small calories per gram of diet)

Identical rat series[1]			*Paired-rat series*		
Rat number	4% protein	12% protein	Pair number	20% protein	42% protein
469	579	269	1	389	720
470	357	246	2	239	439
471	251	99	3	0	632
472	479	464	4	64	490
473	289	261	5	238	522
474	262	251	6	261	317
475	268	158	7	415	436
476	158	133	8	246	378
Average	330	235		232	492
M	95				260
S	95				191
P	0.017				0.0044

[1] The heat increment of the diets used in this test were made on successive days on the same rat, and the order in which the diets were fed was reversed with alternate rats.

Note: Because of lack of space, these are the only individual data presented. The writer will loan copies of the individual data from which the summary tables presented in this publication are based to any one requesting them.

small differences in total heat production were being measured, over 90% of the total heat produced in a test being accounted for by the basal metabolism. However, it was extremely gratifying to obtain differences between pair-mates in a series almost always in the same direction. The individual data for each animal in two different series of tests presented in table 2 will illustrate this point.

Because of a number of possible factors (feed level, previous feed level, composition of the animal, B.M.R., etc.), the most accurate and significant comparisons are those in a single series where controls were most exact and comparable. It is interesting, nevertheless, to calculate the average heat increments of the diets used. This has been done by simply averaging all heat increments obtained on each diet regardless of whether or not they were obtained in different series. These averages, together with the average gross, metabolizable and net energy contents of the various diets, are given in table 3.

TABLE 3

Average heat increments and net energy values of diets containing different percentages of protein

Per cent protein in diet	*Gross energy* [1]	*Metabolizable energy* [1]	*Net energy* [1 3]	*Heat increment* [1]	*Heat increment* [2]	*Net energy* [2]
	Cal.	Cal.	Cal.	Cal.	%	%
4	4641	4412	4040	372	8.43	91.57
8	4722	4382	3980	402	9.17	90.83
12	4752	4429	4194	235	5.30	94.69
18	4800	4507	4372	135	3.00	97.00
20	4848	4624	4465	159	3.44	96.56
30	5083	4702	4544	158	3.36	96.64
42	5238	4754	4264	492	10.35	89.69
54	5480	4842	4217	625	12.90	87.09

[1] Small calories per gram.

[2] Expressed as a per cent of the metabolizable energy.

[3] Calculated by subtracting the average heat increment from the metabolizable energy.

The absolute values given in table 3 are of no great significance, except as they apply to the particular kind of protein used, the amounts of diet consumed and the particular requirements of the animals consuming the diet. The trends, however, are highly significant. For example, the average heat increment of the 8% protein diet was 402 calories per gram, a figure slightly higher than the average of 372 for the 4% diet; however, in the series in which the 4 and 8% protein diets were compared in comparable rats, the heat increments

were 415 for the 4 and 304 for the 8% diet. Forbes, Voris, Bratzler and Wainio ('38) have recently criticized a previously published conclusion from this study (Hamilton, '35, '37) because the average heat increment of the 4% diet was slightly higher than that of the 8% protein diet. The writer believes that the above data show the criticism to be unjust.

As may be seen from the data in tables 1, 2 and 3, the heat increments of the diets fed to growing rats decreased as the percentage of protein increased up to about 18, remained practically constant for diets containing between 18 and 30% protein, then rapidly increased as the percentage of protein increased above 42. The average heat increment, expressed as a per cent of the metabolizable energy, was fairly high, about 9%, for diets containing between 4 and 8% protein; was very low, not much more than 3%, for diets containing between 18 and 30% protein, and was high again, nearly 13%, for the 54% protein diet. A similar trend in the percentage figures is obtained if the gross energy of the diets is used, but unquestionably the loss as heat increment related to the metabolizable energy content is more significant.

Because of some evidence of 'egg white' toxicity, particularly in the rats receiving the higher percentages of the commercial egg protein, the heat increment of a 54% cooked egg protein diet was determined, using four stock rats similar in age, sex and size to those used in previous tests. The heat increment of this cooked protein diet averaged 784 ± 133 calories per gram of diet—a value somewhat higher than the average found for the commercial egg protein diet. It may be assumed, therefore, that the high heat increment observed with the high protein diets was due to the disproportionate amount of protein to other nutrients in the diet, and not appreciably to egg white toxicity.

Comparing the growth-promoting properties of diets containing different percentages of protein with their heat increments, a remarkable inverse relationship is evident. Just as the growth-promoting values of these diets increased as the percentage of protein increased from 4 to at least 16,

and then decreased when the diets contained 42% or more protein (compare preceding paper) the wastages of food energy as heat increment decreased as the percentage of protein increased from 4 to approximately 18 and then rapidly increased in diets with 42% or more protein.

In diets containing extracted dried whole egg as the source of protein and otherwise complete and adequate, the proper balance for the growing rat seems to be obtained when between 16 (probably 20 for young rats) and at least 30 (seemingly between 30 and 42) % protein is present. It is between these two levels that the diets showed the highest growth-promoting values and, at the same time, the smallest waste of energy as heat (heat increment).

The explanation of the effects of the percentage of protein in the diets used in this study upon its growth-promoting value now seems clear. When two diets, one less well balanced with respect to protein than the other, are fed to paired rats in equicaloric amounts, the rat receiving the better balanced diet grew faster and deposited more energy in body gains than did the rat on the less well balanced diet. The reason why the rat receiving the better balanced diet (the one which satisfied more completely the animal's requirements) deposited more energy in his gains than did the animal on an equicaloric amount of the less balanced diet was not because the animal was less active, not because the diet was digested more completely nor because the rat's basal metabolism had been lowered,[2] but primarily, and perhaps solely, because there was less wastage of absorbed energy as heat (heat increment).

The explanation as to why the heat increments of diets decrease instead of increase, as the percentage of protein in the diet increases from 4 or 8 to at least 16, cannot be found in

[2] The calculated energy used for basal metabolic purposes expressed as a per cent of the metabolizable energy intake was, for each series as follows: 4 versus 8 (= food), 68.7 and 78.4; 8 versus 12 (= food), 55.7 and 56.6; 12 versus 16 (= food), 50.3 and 50.7; 4 versus 8 (= gain), 68.4 and 76.8; 8 versus 12 (= gain), 64.8 and 71.8; 12 versus 16 (= gain), 59.6 and 58.6; 8 versus 18 (= gain), 55.1 and 64.3; 16 versus 20 (= gain), 60.5 and 59.6; 18 versus 22 (= gain), 54.9 and 55.2; 22 versus 42 (= gain), 54.2 and 52.0; and 20 versus 54 (= gain), 58.1 and 56.7.

any of the generally accepted theories of specific dynamic effect. Contrary to the present findings, all of the generally accepted theories of specific dynamic effect would predict an increase in the heat increment coincident with every increase in protein consumption, for regardless of several differences of opinion as to the physiological mechanism, protein has always been found to have had a much greater specific dynamic effect than either carbohydrates or fats.

Forbes, Swift, Black and Kahlenberg ('35) working also on the problem of food energy utilization as affected by the percentage of protein, found that the growth-promoting abilities of the diets increased as the percentage of protein increased from 10 to about 20 and they also calculated that, as the percentage of protein increased from 10 to 20, a decreasing amount of energy was either expended as energy of voluntary activity or lost as energy of heating effect (heat increment) (the plan of their experiment making it impossible to differentiate between the two). In an attempt to determine in which of these two ways the decrease was affected, these workers estimated the specific dynamic effects of their diets using previously determined specific dynamic effect factors for proteins, fats and carbohydrates when fed separately (Kriss, Forbes and Miller, '34). By this method they found, naturally, that as the percentage of protein in the diet increased, the estimated heating effect of the diets also increased. Since this was just the opposite of what would be necessary to explain their results, they assumed without measuring it that the activities of the rats must have been voluntarily suppressed sufficiently to account for the observed decrease in total heat production.

In the present study, both the voluntary activities and the heat increments of the diets were directly measured. It was found that as the per cent of protein in the diet increased from a low level to one adequate for rapid growth, the voluntary activity of rats does not decrease and, further, that the heat increments of the diets do decrease as the percentage of protein in the diets increases from a low level to levels adequate for rapid growth.

Concerning diets containing a sufficient excess of protein to lower their growth-promoting abilities, it was found that they had higher heat increments than did well-balanced diets. Also, the voluntary activities of rats receiving a 20% protein diet were greater, not smaller, than were those of rats receiving a 54% protein diet (compare preceding paper).

Forbes, Voris, Bratzler and Wainio ('38) in a recent contribution on this same problem found, in agreement with the present findings, a decrease in the growth-promoting values of diets containing an excess of protein (more than 25% according to Forbes and more than 30 according to the present findings), coincident with an increase in heat production. Again the Pennsylvania workers did not fractionate the energy contributing to the heat production but concluded that the decrease in the quantity of energy utilized for body gain, which is not a measure of total utilizable energy, was due to a more rapid decrease in metabolizable energy than in heat production.

From data presented in the preceding paper, it was concluded that, as the percentage of protein increases above 20, the percentage of the gross energy which was metabolizable, decreased. However, when the quantity of energy lost as heat (heat increment) was actually measured, it was found that the percentage of the metabolizable energy lost increased. For the 20% protein diet, it averaged 3.44; for the 42% protein diet, 10.35, and for the 54% protein diet, 12.90.

Johnson, Hogan and Ashworth ('36) have also studied the effects of different levels of protein intake upon the utilization of food energy. These workers found greater rates of gain by rats on diets containing 26% protein than on diets containing 10% protein. The plan of their experiments, however, enabled them to study the partition of the metabolizable energy only with respect to the energy stored in the gains. They report no difference in the total energy stored on the two diets, but, in agreement with others, that the rats on the low protein diet stored more fat and less protein than did the rats on the higher protein intake.

But the energy stored is only one way in which the metabolizable energy of a diet is utilized, and a measurement of the extent to which the metabolizable energy is retained in the body is not a complete measure of the extent to which the energy of a diet is utilized by the body.

The utilizable energy of a diet is that fraction of the metabolizable energy which can be used in supplying energy for 1) basal metabolism, 2) voluntary activity and 3) productive purposes. The summation of these fractions constitutes the net energy of the diet. An accurate comparison of the efficiency with which the energy of different diets is utilized cannot be made without including all three of these fractions. Since it is very difficult, if not impossible, to measure accurately the energy of voluntary activity, the most feasible method of estimating the net energy of a diet is by determining the heat increment and subtracting it from the metabolizable energy. This was the procedure followed in the investigation here reported.

The net energy, measured as the metabolizable energy minus the heat increment (equal to the total increase above the fasting level in the heat production incident to the consumption of the diet), was found to vary directly, and the heat increment inversely, with the growth-promoting values of the diets. These findings are in harmony with previous observations referred to above to the effect that the specific dynamic effects of the food nutrients were less when the requirements of the animals for the nutrients were greatest, and also that diets better balanced with respect to other essential nutrients will promote more rapid growth rates than will an equicaloric amount of less well-balanced diets.

The data support the conception that the better balanced a diet is in satisfying an animal's requirements, the smaller will be its heat increment and the greater will be its net energy value.

CONCLUSIONS

Increasing the percentage of protein from 4 to 54 by direct substitution of dried, extracted whole egg for starch, in the

diet of growing male rats, had the following effects upon the utilization of the dietary energy:

1. The heat increment (specific dynamic effect) of the diet decreases coincident with every increase in the percentage of protein until about 18% is present; it remains constant in diets containing between 18 and at least 30% protein; and then increases again in diets containing 42% or more protein.

2. There is a remarkable inverse relationship between the heat increments and the growth-promoting abilities of the diets.

3. The net energy value of diets for growth plus maintenance increased as the percentage of protein in the diet increased up to about 16%, remained practically constant throughout the range of protein percentages over which the diets were equally well balanced (16 to 30%) and then decreased rapidly when an excess of protein was present (perhaps more than 30% whole egg protein).

4. The explanation of the higher net energy value of well-balanced diets, as compared with diets unbalanced either because of an insufficiency or because of an excess of protein, lies almost entirely in the smaller heat increment of the well-balanced diets.

5. The percentages of metabolizable energy used for basal expenditures or for total voluntary activity are unaffected by the percentage of protein in the diet, independent of the amount of food consumed.

The findings in this investigation support the general conception that the better balanced a diet is in satisfying an animals requirements, the smaller will be its heat increment and the greater will be its net energy value.

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A STUDY OF RACHITOGENIC DIETS COMPOSED OF PURIFIED FOOD MATERIALS [1]

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ONE FIGURE

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In order to produce rickets in rats it is not only necessary to have a diet which is deficient in the antirachitic vitamins but it must also be low in phosphorus. In the past such diets, for the greater part, have been composed of cereal grains such as yellow maize (Steenbock and Black, '25) or wheat (McCollum et al., '21; Sherman and Pappenheimer, '21). These grains owe their rachitogenic properties to the fact that they are low in phosphorus and a considerable portion of that present is not available for the rat (Bruce and Callow, '34). In addition it has been found necessary to decrease further the availability of the phosphorus by the addition of excess calcium carbonate. The advantages of a purified or of a so-called synthetic rachitogenic diet over the cereal diets are numerous. For example, it has been shown that the Steenbock-Black rachitogenic diet is deficient in vitamin A, protein and vitamin B complex (Kletzien et al., '32; O'Brien and Morgareidge, '38). It should be possible to correct these deficiencies with a synthetic diet. If the total amount of phosphorus is materially reduced it would be unnecessary to rely upon the unavailability of a portion of that present to produce rickets, and also it would allow for the production of severe rickets with greater regularity and possibly in a shorter

[1] Presented before the biochemical section of the American Chemical Society, Milwaukee, September 5 to 9, 1938.

time. Such a diet could also be reproduced with greater constancy than the cereal diets, because cereals vary in their content of both total and available phosphorus (Snook, '38), and, aside from its content of phosphorus, maize varies in its rachitogenic properties (Harris and Bunker, '33, '34). Furthermore, a diet composed of individually purified constituents would lend itself to the study of the various factors concerned in calcium and phosphorus metabolism much better than diets composed of natural food materials. The greatest difficulty in the preparation of a purified diet low in phosphorus has been to obtain readily a satisfactory and inexpensive protein. Casein which has been used so widely in synthetic diets is not suitable as it contains too much phosphorus. Several years ago Osborne, Mendel and Park ('23) described a purified rachitogenic diet containing either edestin or lactalbumin as the protein. However, possibly due to the proteins and also to the complete absence of vitamin A their diet was never used extensively. Recently we reported (Jones, '38) that in our search for a protein low in phosphorus we had found that blood-fibrin met the desired requirements. With fibrin as the purified protein a number of rachitogenic diets have been constructed and studied, some of which are presented at this time.

EXPERIMENTAL

Rats were used as test animals and were usually placed on the experimental diet when 25 days of age. Unless otherwise stated, 21 days later they were bled under ether anesthesia and the bloods from several rats (usually three) were united and the sera analyzed for calcium by the method of Clark and Collip ('25) and for inorganic phosphorus on the calcium-free filtrate by the method of Gunther and Greenberg ('29). The right femur was removed from each animal for ash analysis and the wrist bones were taken for examination by the line-test technic.

The composition of a number of the experimental diets and the phosphorus content of each are given in table 1. Salt

mixture no. 5, which was used in several of the diets, is composed of the following ingredients expressed in relative parts: NaCl 1, $CaCO_3$ 3, KCl 1, $MgSO_4$ (anhydrous) 0.2, ferric citrate 0.1, and KI 0.004. When fed at a level of 5.3% of the ration this salt mixture furnished 1% NaCl and 3% calcium carbonate, which is the same as that in the Steenbock and Black ('25) and McCollum et al. ('21) rachitogenic diets. Diet 570 which has proved very successful is composed of alcohol-extracted fibrin 18%, brewers' yeast 5, salt mixture

TABLE 1

Percentage composition of several purified rachitogenic diets

Diet no.	570	573	610	576	611	612	618	623
Fibrin	18	18	18	18	18		18	18
Yeast	5	5		5			5	5
Salt no. 5	5.3	5.3	5.3	5.3	5.3	5.3	5.3	
Agar	2	2						
Carotene solution	0.1	0.1	0.1	0.1	0.1	0.1		
Dextrin	69.6						69.7	71
Sucrose		69.6	73.6	71.6	73.04	78.8		
Liver extract 343			1.0		1.0	1.0		
Thiamine			20[1]		20[1]			
Cellulose			2.0		2.0	2.0		
Casein						12		
Yeast concentrate						0.8		
K_2HPO_4					0.56			
Alfalfa							2	2
NaCl								1
$CaCO_3$								3
Total phosphorus	0.08	0.08	0.02	0.08	0.12	0.14	0.10	0.10

[1] Micrograms per day.

no. 5, 5.3, agar 2, carotene solution (Mead Johnson and Co.) 0.1 and dextrinized starch 69.6. The starch was dextrinized in the laboratory by autoclaving. It is necessary that the starch contain not more than traces of phosphorus. In diet 573 the dextrin was replaced by sucrose. In most cases in which sucrose comprised a large part of the ration the rats consumed insufficient food for the first 2 or 3 days to maintain their weight. This apparent reduction of appetite was of short duration, and with an increase in food consumption

the animals showed considerable gain in weight. Diets 570 and 573 contain about 0.08% phosphorus and both produced severe rickets in 3 weeks or less.

The greater part of the phosphorus of these two diets is supplied by the yeast. Likewise, aside from the 0.1% carotene solution (and in some cases crystalline carotene was used) the only unpurified materials in diet 573 are the yeast and agar. Consequently in diet 610 (table 1) a higher degree of purity was obtained and the amount of phosphorus was simultaneously reduced by replacing the agar with an equal amount of regenerated cellulose[2] and supplying the B factors with 1% Lilly Liver Extract 343 and 20 micrograms of thiamine[3] daily. By so doing the phosphorus of the diet was reduced to 0.02%. This diet also produced marked rickets in a short time. In figure 1 are shown sections of a wrist bone from one animal on each of these three diets taken at the end of 3 weeks and prepared with silver nitrate in the usual manner. As can be seen the rachitic condition is very marked and the margins between the calcified and uncalcified areas are straight and regular. There is a complete lack of the so-called 'cupping' or extension of the calcified area along the margin of the bones. In some cases the degree of rickets was not as marked as that illustrated, but without exception well-defined rickets was obtained. Omission of the roughage as in diet 576 had no effect on the severity of the resulting rickets.

Having shown that it is possible to produce severe rickets on a highly purified diet, indicating that the only factors concerned with the production of rickets are vitamin D and the amount and availability of phosphorus, we next turned our attention to the effect of adding phosphorus to the diet on the development of rickets. When the phosphorus content of diet 610 was increased to 0.12% by the addition of 0.56% dipotassium phosphate (diet 611, table 1) the degree of rickets produced was less as indicated by an extension of the calcified

[2] Kindly sent to the author by Dr. C. M. McCay of Cornell University.
[3] Furnished by Merck and Company, Inc., Rahway, N. J.

area along the margin of the bone or the 'cupping' referred to above (fig. 1).

A short time ago Harris and Bunker ('37) reported that they were unable to obtain rickets in rats on a diet containing casein even though the casein had been partially dephosphorized. Their diet, however, still contained about 0.2% phosphorus which, judging from our results, would be too high

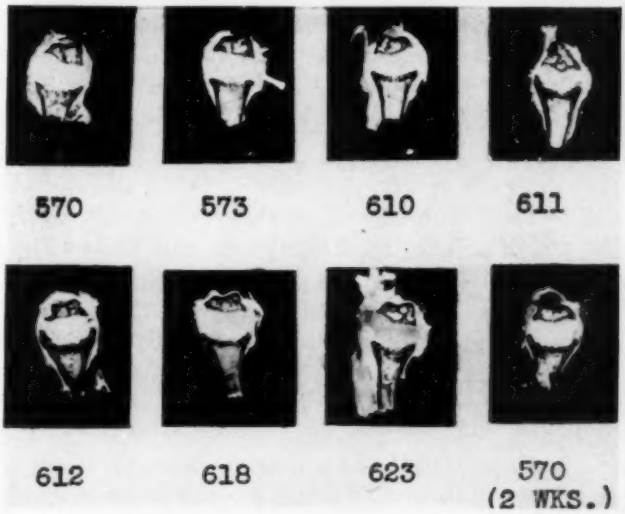

Fig. 1 Production of rickets on various diets. Numbers refer to diets described in text.

to allow for the production of rickets. Consequently an effort was made to reduce the phosphorus in a casein-containing diet considerably below this level. No steps were taken to remove the phosphorus chemically combined as part of the casein molecule, but purified casein[4] was fed at a level of 12% as indicated in diet 612 (table 1). The vitamin B factors were supplied with 1% liver extract and 0.8% Harris yeast concentrate. Such a diet contains about 0.14% phosphorus

[4] Supplied by Smith, Kline and French, Inc., Philadelphia, Pa.

and very definite rickets was produced. However, as shown in figure 1 there was some 'cupping' and the results were very similar to those obtained on diet 611 which had approximately the same amount of phosphorus. Similar results have been obtained with several different diets in which casein has been used as the protein. It thus appears that aside from its content of phosphorus casein has no special antirachitic properties. Under the conditions prevailing in this laboratory a rachitic picture quite like that resulting from feeding diets 611 and 612 has been frequently obtained on the Steenbock-Black diet ('25). From this it would seem there is probably from 0.10% to 0.15% of available phosphorus in this ration.

In an attempt to construct as inexpensive and simple a diet as possible the vitamin A and roughage of diet 570 were supplied by 2% of alfalfa, as shown in diet 618 (table 1). The phosphorus content of this diet is approximately 0.1% and very good rickets was obtained in 3 weeks (fig. 1). It was also found possible to reduce the fibrin to 12% without influencing the results. As the salt mixture used in these diets is somewhat more complex than that used by either Steenbock or McCollum, diet 618 was modified to contain 1% sodium chloride and 3% calcium carbonate in place of salt mixture no. 5, as shown in diet 623. Again a marked degree of rickets was produced (fig. 1).

In doing routine work it is desirable to shorten the length of each procedure as much as possible. In the last photograph of figure 1 is shown the results of feeding diet 570 for only 2 weeks instead of 3 weeks. It is obvious that on this diet well-advanced rickets can be obtained in no more than 14 days and possibly less. Recently Schneider and Steenbock ('38) have reported a diet low in phosphorus on which they were able to produce rickets in rats in a period of 2 weeks. As their diet contained 18% cooked egg white and 4% Vitab it cannot be classed as a purified diet. Furthermore, it is rather costly and somewhat laborious to prepare.

In table 2 are given the gains in weight of the animals, the ash content of the femurs and the level of calcium and inorganic phosphorus of the sera. The results are given as the averages for each group. For the most part, the rats gained about 25 gm. in weight during the experimental period of 3 weeks. When liver extract and thiamine replaced yeast as in diets 610 and 611 the gains were considerably less. Indications are that some of the B factors were not being supplied in sufficient quantity. In other experiments in which casein was the protein growth was below normal when the B complex was supplied as above and the diet was otherwise complete.

TABLE 2

Effect of various rachitogenic diets on femur-ash and calcium and phosphorus of serum

DIET NO.	NUMBER OF ANIMALS	GAIN IN WEIGHT	FEMUR ASH		SERUM		
					Number of determinations	Ca[1] mg. per 100 cc.	P[1] mg. per 100 cc.
		gm.	mg.[1]	%[1]			
570	27	22.9	16.4	21.7	9	12.8	2.3
573	24	22.6	18.6	23.6	8	13.2	2.6
610	10	9.5	15.9	23.5	3	13.1	2.6
576	6	20.3	15.9	22.1	2	12.6	2.1
611	3	12.7	27.5	36.1	1	13.2	3.1
612	3	21.3	27.1	31.7	1	13.6	2.6
618	6	32.2	16.9	23.9	2	12.7	2.4
623	4	27.7	17.4	21.6	1	13.0	2.2

[1] Average of number of animals indicated.

Furthermore by increasing the liver extract good growth was obtained with fibrin but because it was desired to keep the phosphorus content of these rations as low as possible the level of liver extract was not increased in these experiments. There is little difference in the ash content of the femurs of the various groups except those on diets 611 and 612. These diets as stated above were higher in phosphorus and produced less severe rickets as shown by examination of the wrist bones. This increased calcification is reflected in both the amount and percentage of femur ash. In respect to the

serum, the inorganic phosphorus is considerably below normal and the calcium somewhat above. The phosphorus of the group of rats on diet 612 is slightly below that expected but of several groups on similar diets containing casein this was the lowest value for phosphorus obtained. As might be predicted the phosphorus of the serum of the rats given the extra phosphorus in their diet (diet 611) was a little above that of the others.

The above data demonstrate that it is possible to produce rickets in rats on a diet composed almost entirely of purified food substances if the phosphorus content is maintained at a low level. This furnishes additional evidence in favor of the view that cereals do not owe their rachitogenic properties to the presence of a specific anti-calcifying agent. Also it gives a better opportunity for a study of the factors leading to the development of rickets in the rat. Whether such diets can be used to advantage in the routine assay of materials for their vitamin D content remains to be determined.

SUMMARY AND CONCLUSIONS

A number of low-phosphorus diets rachitogenic for the rat and composed of purified food material have been described. Blood fibrin was used as the protein in most of the diets. A fair degree of rickets can be obtained with casein if fed at a level of not over 12%. Apparently the antirachitic activity of casein is due entirely to the large amount of phosphorus contained in this protein. In order to produce severe rickets in the rat on diets containing 3% $CaCO_3$ the available phosphorus must not be greater than approximately 0.1%.

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